A decade of experience emphasizes that testing for Y microdeletions is essential in American men with azoospermia and severe oligozoospermia

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Objective: To evaluate the benefit of Y microdeletion testing.
Design: Retrospective analysis.
Setting: University-based male fertility clinic and genetics laboratory.
Patient(s): A total of 1,591 men with sperm concentrations less than 5 million sperm/mL.
Intervention(s): Semen analysis, Y microdeletion testing, microdissection testicular sperm extraction (TESE).
Main Outcome Measure(s): Sperm concentration, incidence and nature of Y microdeletions, microdissection TESE outcome.
Result(s): We identified 149 microdeletions (9.4%). 10.4% of azoospermic men and 10.1% of men with sperm concentrations >0 1 million sperm/mL harbored microdeletions. Two-thirds of microdeletions in azoospermic men were AZFa, AZFb, AZFb+c, or complete Yq deletions. Virtually all microdeletions in oligozoospermic patients were AZFc deletions. Seven hundred eighteen patients underwent microdissection TESE, including 41 with microdeletions. Microdissection TESE failed in all patients with AZFa, AZFb, AZFb+c, and complete Yq deletions. Sperm were retrieved in 15/21 AZFc deleted patients (71.4%). The presence of an AZFc deletion was associated with increased likelihood of sperm retrieval when compared with the 48.8% retrieval rate in 385 idiopathically azoospermic men who consecutively underwent microdissection TESE at our institution during the study period. Clinical pregnancy was achieved in 10/15 azoospermic AZFc deleted patients for whom sperm were successfully retrieved.
Conclusion(s): Of azoospermic and severely oligozoospermic American men, 10% harbor Y microdeletions that alter prognosis for surgical sperm retrieval and are vertically transmissible. Y microdeletion testing is essential for genetic and preoperative counseling in these patients. (Fertil Steril® 2010;94:1753 6. ©2010 by American Society for Reproductive Medicine.)

Key Words: Male infertility, azoospermia, oligozoospermia, Y microdeletions, testicular sperm extraction, sperm retrieval, Y chromosome, genetic testing

Interstitial deletions that occur in the azoospermic factor (AZF) region of the long arm of the Y chromosome (Yq) are referred to as Y chromosome microdeletions. Y microdeletions account for up to 20% of cases of severe idiopathic male infertility (1) and are a leading genetic cause of male factor infertility worldwide. Elegant work performed during the past decade has elucidated the complicated structure of the male-specific portion of the Y chromosome and the de novo genetic events from which Y microdeletions result (2 4).

Similar to all couples considering using advanced reproductive technologies (ART), those affected by Y microdeletions must weigh the significant psychological, financial, and medical costs of undergoing treatment against the chances of achieving pregnancy. They must also consider that sons conceived with sperm from men with Y microdeletions are expected to inherit the abnormal Y chromosome and the subfertile phenotype (5, 6). Most reproductive endocrinologists and male fertility specialists agree that testing for Y microdeletions is an essential part of the evaluation of subfertile severely oligozoospermic or azoospermic men, and testing is recommended by the American Society for Reproductive Medicine (ASRM) practice guidelines (7, 8). Nonetheless Y microdeletion testing is not universally offered to couples affected by severe oligozoospermia or azoospermia (9).

One reason for heterogeneity in Y microdeletion testing practices throughout the world is the well-documented geographic variability in Y microdeletion frequencies that has been observed. In the largest screening studies published to date, the frequencies of Y microdeletions among severely oligozoospermic men from Germany and Italy were 1.8% and 5%, respectively (10, 11). Smaller studies from Australia, Scandinavia, China, India, Japan, and Russia have revealed Y microdeletion frequencies that range from 1% 14% (10). American studies have been limited by small patient populations and have found highly variable microdeletion frequencies that have ranged from 3% 18% (10). In geographic regions where Y microdeletions are less common, the cost effectiveness of testing is diminished and the necessity of testing is less clear.

Received June 4, 2009; revised July 31, 2009; accepted September 8, 2009; published online November 6, 2009.
P.J.S. has nothing to disclose. P.M. has nothing to disclose. A.M. has nothing to disclose. M.B.M. has nothing to disclose. P.N.S. has nothing to disclose. D.A.P. has nothing to disclose.
Presented at 2008 meeting of the American Society for Reproductive Medicine, San Francisco, CA, November 8–12, 2008.
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Other arguments commonly cited against routine Y microdeletion testing of severely oligozoospermic men are that diagnosis of Y microdeletions usually does not alter the prognosis or treatment of affected patients, and that interinstitutioual methodological variability in Y microdeletion testing makes it difficult to draw conclusions about the clinical utility of testing. Furthermore, widespread access to intracytoplasmic sperm injection (ICSI) using ejaculated or extracted spermatozoa has euabled some practitioners treating affected couples to proceed with IVF without formal evaluation of the male partner.

We do know that men with complete deletions of AZFa, AZFb, and AZFb+c are universally azoospermic without significant hope of testicular sperm retrieval (12, 13). Diagnosis of these Y microdeletions saves patients the potential morbidity of attempted surgical sperm retrieval and euables them to promptly consider using donor sperm or adoption. However, AZFa, AZFb, and AZFb+c microdeletions are relatively rare in azoospermic men with a reported collective prevalence of 4.4% in a large series of azoospermic men screened in Italy (11).

The AZFc microdeletion is by far the most commonly encountered Y microdeletion, comprising 60% of all AZF deletions detected (14). Affected men have variable spermatogenic phenotypes that range from oligozoospermia to complete germ cell absence. Our ability to draw meaningful conclusions about the prognostic relevance of the AZFc deletion has been limited by very small numbers of patients in the existing reports of attempted sperm retrievals. To date no study has demonstrated a statistically significant prognostic benefit to testing for the AZFc deletion in azoospermic men considering surgical sperm retrieval.

We have previously reported our initial experience with surgical sperm retrieval and IVF/ICSI in men with Y microdeletions (12, 13). However, we have yet to report the frequency of Y microdeletion detection among severely oligozoospermic and azoospermic men in our patient population. Furthermore, we have performed considerably more microsurgical sperm retrievals on both deleted and nondeleted azoospermic patients since our prior publications and can now make meaningful statistical assessments of the prognostic relevance of Y microdeletions.

In the present study we report a decade of experience in the diagnosis and surgical management of men with Y microdeletions. Our series of consecutively screened patients is one of the largest reported worldwide, and the single largest series reported from the United States. Furthermore, our series of attempted microdissection testicular sperm extractions (TESE) in men with Y microdeletions is the largest in the literature reported to date.

MATERIALS AND METHODS

The Institutional Review Board (IRB) of the Weill Cornell Medical College approved this study. The study population was an ethnically heterogeneous cohort of 1,591 subfertile men, with sperm concentrations less than 5 million sperm/mL, screened consecutively for Y microdeletions at our laboratory from 1997–2007. Three hundred seventy-six patients were clinically evaluated and treated at other centers throughout the United States. For these patients a blood sample was sent to our laboratory for Y microdeletion testing, along with results of semen analyses. The remaining 1,215 patients were clinically evaluated and treated at our institution. All subjects consented to genetic testing. Sperm concentrations, microdissection TESE outcomes, and clinical pregnancy results were retrospectively reviewed. For patients with multiple semen analyses, the median of individual sperm concentration results was calculated and recorded.

Y microdeletion screening was performed according to previously published methods (13, 15) by multiplex polymerase chain reaction (PCR) of DNA extracted from peripheral blood leukocytes. For each patient genomic DNA was extracted from peripheral blood using two methods, the Stratagene DNA Extraction Kit (Stratagene, La Jolla, CA) and the Genomic DNA Purification Kit (Promega, Madison, WI). Thirty sequence-tagged sites (STS) within the AZF region of Yq11 and the SRY gene (sY14) were targeted for PCR amplification. All patients were screened twice with multiplex PCR using different DNA sources. DNA from a fertile man served as a positive control. Water and DNA from a woman were used as negative controls. To confirm multiplex PCR results that indicated a Y microdeletion, single primer PCR analyses were performed in duplicate for all deleted STSs and two flanking STSs. Given the lack of convincing data regarding the clinical relevance of partial deletions of the AZFc region, patients with partial AZFc deletions were considered nondeleted in this analysis.

Microdissection TESE was performed by a single surgeon, as has previously been described (16). A semen specimen was obtained immediately before planned microdissection TESE to confirm azoospermia. If no sperm were identified within the pellet produced by centrifugation of the sample at 3,000 × *g* for 15 minutes, microdissection was performed using the operating microscope and one transverse incision in the tunica albuginea through which the entire volume of testicular tissue was dissected until sperm were found.

Microdissection TESE outcomes in patients with microdeletions were compared with outcomes in men with idiopathic azoospermia who consecutively underwent microdissection TESE at our institution during the study period. Idiopathic azoospermia was defined as azoospermia in the absence of cytogenetic or genetic abnormalities, Klinefelter's syndrome, a history of cryptorchidism, congenital hypogonadism, obstructive azoospermia, history of chemotherapy, or history of pelvic irradiation. For patients in whom sperm were successfully retrieved, pregnancy outcomes of IVF/ICSI cycles were reviewed. ICSI was performed in all cases with fresh spermatozoa. Clinical pregnancy was established by the presence of fetal heartbeats determined by transvaginal ultrasonography (TVUS) approximately 32 days after embryo transfer.

Differences in microdissection TESE and clinical pregnancy rates (PR) between patients with microdeletions and patients with idiopathic azoospermia were evaluated for statistical significance using the Fisher's exact test.

RESULTS

Of 1,591 severely oligozoospermic and azoospermic men tested, we identified 149 subjects with Y microdeletions (9.4%). The specific microdeletion status, the number of men found to have each deletion, and the results of their semen analyses are depicted in Table 1. One hundred twenty of 1,193 azoospermic men (10.4%) and 26 of 257 severely oligozoospermic men with sperm concentrations less than 1 million/mL (10.1%) were diagnosed with a Y microdeletion, whereas only 3 of 181 men with 1 5 million sperm/mL (1.7%) were positive for microdeletions. All men with complete AZFa, AZFb, and AZFb+c, and AZFa+b+c deletions were azoospermic. One patient with a partial AZFb+c microdeletion, in which the centromeric portion of the AZFb region was not deleted, had sperm in his ejaculate. Seventy-eight study patients had AZFc deletions (4.9%). The AZFc deletion was particularly prevalent in severely oligozoospermic men with sperm concentrations between 0 and 1 million sperm/mL, found in 9.7% of these patients.

Ontcomes of microdissection TESE in this patient population are presented in Table 2. Seven hundred eighteen azoospermic study patients underwent microdissection TESE, including 41 patients with Y microdeletions and 385 patients with idiopathic azoospermia. Microdissection TESE universally failed in men with AZFa, AZFb, AZFb+c, and complete Yq deletions. Microdissection TESE was successful in 15 of 21 patients with complete AZFc deletions (71.4%). Microdissection TESE was more likely to succeed in AZFc deleted patients than in nondeleted men with idiopathic azoospermia (P=.035), in whom the surgical sperm retrieval rate was 48.8%.

TABLE 1

Prevalence of Y microdeletions among subfertile severely oligozoospermic and azoospermic men stratified by sperm concentration.

Sperm concentration (million/mL)	Total screened	AZFa (%)	AZFb (%)	AZFb+c (%)	AZFc (%)	Complete Yq (%)	Any Y microdeletion (%)
Azoospermic	1,153	4 (0.3%)	17 (1.4%)	31 (2.7%)	50 (4.3%)	18 (1.6%)	120 (10.4%)
>0–<1	257	0	0	1 (0.4%)[a]	25 (9.7%)	0	26 (10.1%)
1–<5	181	0	0	0	3 (1.7%)	0	3 (1.7%)
Total	1,591	4 (0.3%)	17 (1.1%)	32 (2.8%)	78 (4.9%)	18 (1.1%)	149 (9.4%)

[a] Partial AZFb+c deletion that spared the centromeric portion of the AZFb region.

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Clinical pregnancy outcomes of IVF/ICSI cycles for couples in whom sperm were successfully retrieved are presented in Table 3. Clinical pregnancies were achieved in 91/188 patients with idiopathic azoospermia (48.4%) and in 10/15 AZFc deleted patients (67.7%) (nonsignificant, P=.19).

DISCUSSION

Review of our experience with Y microdeletions re-emphasizes the importance of testing for Y microdeletions in severely oligozoospermic and azoospermic men. For both groups of patients, Y microdeletions are sufficiently prevalent in the United States to justify routine testing in couples considering assisted fertility.

We diagnosed Y microdeletions in 26/257 (10.1%) severely oligozoospermic men with sperm concentrations more than 0 but less than 1 million sperm/mL. This is slightly higher than the 6% 8% reported prevalence of Y microdeletions in severely oligozoospermic men tested and treated at other centers throughout the world (10, 11, 17, 18). The significant prevalence of Y microdeletions in severely oligozoospermic American patients underscores the necessity to perform Y microdeletion screening in these men, even in cases where enough spermatozoa for ART have already been collected. Failure to do so compromises the treating physician's ability to adequately counsel these patients before ART about the risks of subfertility in their male offspring.

TABLE 2

Outcomes of microdissection TESE in azoospermic men stratified by Y microdeletion status.

Etiology of azoospermia	Sperm retrieved	Sperm not retrieved	Total	Retrieval rate
AZFa	0	2	2	0%
AZFb	0	7	7	0%
AZFb+c	0	7	7	0%
AZFa+b+c	0	4	4	0%
AZFc	15	6	21	71.4%[a]
Nondeleted, idiopathic	188	197	385	48.8%[a]

Note: TESE testicular sperm extraction; AZF azoospermic factor.

[a] Comparison of retrieval rates in AZFc deleted men and idiopathically azoospermic nondeleted men, P<.05 (Fisher's exact test).

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In azoospermic men Y microdeletion testing not only provides essential information for genetic counseling, but it helps patients and their physicians make more informed decisions about surgical sperm retrieval. In this study the prevalence of Y microdeletions in patients with nonobstructive azoospermia was 10.4%. Studies from other geographic areas have reported Y microdeletions in 3% 15% of azoospermic patients (10, 11, 17, 18). Six percent of azoospermic men that we tested were found to harbor microdeletions now established as incompatible with paternity (AZFa, AZFb, AZFb+c, or AZFa+b+c) (12, 13). Although we report only microdissection TESE results, we have seen no men with complete deletions of the AZFa or AZFb regions that had sperm found on biopsy samples of testis or standard TESE. Clinically, we now recommend primary use of donor sperm rather than TESE for men with deletions that involve complete loss of the AZFa or AZFb regions. For these unfortunate patients, Y microdeletion testing avoids the potential morbidity of TESE and clarifies the need for donor sperm or adoption.

We foond AZFc deletions in 4.4% of azoospermic patients tested. Our data are the first to demonstrate that these patients enjoy a statistically better microsurgical sperm retrieval rate (71%) when compared with nondeleted, idiopathically azoospermic men (48%). The idiopathic azoospermia cohort excludes patients with identifiable conditions that have a higher rate of sperm retrieval with microdissection TESE including Klinefelter's syndrome (retrieval rate 68%) (19) and cryptorchidism (retrieval rate 74%) (20). In our experience this information has been useful when preoperatively counseling couples considering microdissection TESE.

To our knowledge, the tested population reported in the present study is the largest cohort of subfertile men that has been tested

TABLE 3

Clinical pregnancy outcomes in idiopathically azoospermic men and in azoospermic AZFc deleted men in whom sperm were surgically retrieved.

Etiology of azoospermia	Sperm retrieved	Clinical pregnancies	Clinical pregnancy rate
Idiopathic	188	91	48.4%[a]
AZFc deletion	15	10	66.7%[a]

[a] Not significantly different (P .19, Fisher's exact test).

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for Y microdeletions in the United States, and our experience with microdissection TESE in men with Y microdeletions is the largest such series published to date. Nonetheless, the ability of our findings to be generalized may be limited. Our institution specializes in the genetic evaluation and treatment of male factor infertility. The high incidence of Y microdeletions we observed might reflect selection bias due to our referral pattern. In addition, as a tertiary care center for men with severe infertility, our screened population of infertile men may be more phenotypically severe than men treated at other centers. This is reflected by the high percentage of azoospermia (70%) in our screened population. Fiually, our sperm retrieval rate of 71% in azoospermic men with AZFc deletions is higher than the retrieval rates reported by other centers, which range from 28% 55% (12, 13). This variability in retrieval rates likely reflects differences in retrieval techniques used at different centers (i.e., use of the operating microscope and extent of testicular dissection).

Despite these limitations we believe that our study emphasizes the need for Y microdeletion testing in severely oligozoospermic and azoospermic American men. Ten percent of these patients harbor Y microdeletions. Diagnosis of Y microdeletions is critical for preconception genetic counseling and provides valuable prognostic information to couples considering surgical sperm retrieval.

Acknowledgment: The authors thank Dr. Marc Goldstein, clinical and scientific adviser.

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Systems Biology in Reproductive Medicine

ISSN: 1939-6368 (Print) 1939-6376 (Online) Journal homepage: www.tandfonline.com/journals/iaan20

A novel sorting technology allows for highly efficient selection of sperm without chromatin damage

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To cite this article: Michael G. Funaro, Howard H. Kim, Svetlana Mazel, Alexander Bolyakov, Marc Goldstein, Peter N. Schlegel & Darius A. Paduch (2013) A novel sorting technology allows for highly efficient selection of sperm without chromatin damage, Systems Biology in Reproductive Medicine, 59:3, 172-177, DOI: 10.3109/19396368.2013.777135

To link to this article: https://doi.org/10.3109/19396368.2013.777135

Systems Biology in Reproductive Medicine, 2013, 59: 172–177

ISSN 1939 6368 print/1939 6376 online
DOI: 10.3109/19396368.2013.777135

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healthcare

APPLICATION NOTES

A novel sorting technology allows for highly efficient selection of sperm without chromatin damage

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Sperm chromatin damage has been associated with male infertility, increased risk for spontaneous abortion, and poor embryo development. Available methods for detecting chromatin damage render the sperm no longer suitable for clinical use. Early apoptotic events resulting in chromatin damage are associated with increased permeability of the cell membrane to large ions. We propose the use of a large fluorescent organic cation, proprietary fluorochrome (PF-1), for fluorescence-activated cell sorting (FACS) for negative selection of sperm without chromatin damage. Sperm with chromatin damage are PF-1 positive. Performance of cell sorting by PF-1 was verified with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) after FACS on PF-1(+) and PF-1(-) subpopulations. Whereas 19.5% of PF-1 positive sperm were TUNEL positive only 1.5% sperm in the PF-1(-) fraction were TUNEL positive ($p < 0.00001$). TUNEL values below 1.9% were considered background fluorescence. Post-sorting motility and vitality were 49.4% (SD: 12.5) and 65.0% (SD: 14.99), respectively. Proprietary fluorochrome activated sperm sorting may decrease or most likely eliminate all of TUNEL positive sperm without adverse effects on viability, providing a new therapeutic avenue for men with a high percentage of TUNEL positive sperm. Further research is needed to determine if the reduction in TUNEL positive sperm using PF-1 will improve *in vitro* fertilization (IVF) outcomes.

Keywords apoptosis, chromatin damage, DNA integrity, flow cytometry, sperm

Abbreviations FACS: fluorescence-activated cell sorting; PF-1: proprietary fluorochrome; FC: flow cytometry; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling; IVF: *in vitro* fertilization; SCSA: sperm chromatin structure assay; Ch: chromosome; ICSI: intracytoplasmic sperm injection; AV: annexin-V; PS: phosphatidylserine; ART: assisted reproductive technologies; PI: propidium iodide;

Introduction

Sperm DNA integrity is recognized as a significant parameter of sperm quality and affects the outcome of assisted reproductive procedures. Studies have demonstrated an association between sperm chromatin integrity and male fertility potential [Zini et al. 2001]. There are reports of an inverse relationship between the percentage of sperm with abnormal chromatin and male fertility, especially when the proportion of aberrant cells, as measured by the sperm chromatin structure assay (SCSA), are greater than 40% [Spano et al. 2000]. Sperm chromatin damage may subject couples to the risk of poor embryo development or recurrent spontaneous abortions [Brahem et al. 2011; Kumar et al. 2012]. However, the interaction of abnormal sperm chromatin integrity and DNA strand breaks that normally occur during DNA compaction is poorly understood. Epigenetic alterations, Y chromosome (Ch) and autosomal Chs microdeletions, and errors in mismatch repair mechanisms contribute to compromised fertility [Tarozzi et al. 2007]. Beyond the immediate effects of the DNA damage, it is also not apparent how DNA integrity assays can be effectively used in clinical practice [Holt and Van Look 2004; Sharma et al. 2004].

In IVF, selection of the sperm is based on motility and morphology without insight into the DNA structure, sperm surface receptor expression, and other biological properties of sperm. In nature, biological sperm selection mechanisms end up rejecting all but very few spermatozoa that are released with ejaculation [Manning and Chamberlain 1994]. The existence of large numbers of spermatozoa may be seen as a compensatory mechanism for inexorable errors and defects in sperm. While the competitive nature of sperm selection is largely removed with intracytoplasmic sperm injection (ICSI), simulating the natural selection of sperm may not provide optimal IVF outcomes and prevent

Received 27 August 2012; accepted 30 January 2013.

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problems with embryo development. It is presently unclear how the events in the female reproductive tract preceding fertilization and the egg itself screen for the biological quality of sperm including DNA quality [Henkel 2012]. It is possible that early changes in sperm membrane are used by nature as proxy for sperm DNA integrity.

There are presently several sperm DNA evaluation procedures used with sperm in assisted reproductive therapies. None of such DNA integrity procedures permits sperm to be used clinically subsequent to analysis. Other sperm selection techniques that preserve sperm largely fail to look at DNA quality in sperm. These methods range from selective microscopic examinations to computer-assisted sperm motility analysis. These methods tend to sort the sperm on significant external properties or defects, and therefore turn a blind eye to DNA integrity and chromatin damage, which arguably may be important traits in the context of IVF.

Available methods for detecting sperm chromatin damage include sperm chromatin structure assay (SCSA), TUNEL, Halosperm, and Comet assays [Dugum et al. 2011]. While these assays are able to test for DNA integrity, they require permanent fixation of the sperm. Permanent fixation of the sperm renders them no longer suitable for clinical use; therefore, these assays are consumptive [Henkel 2012]. In this, there is no opportunity to select between sperm with varying degrees of chromatin damage and select those most suitable for immediate use with IVF or cryopreservation if necessary.

Another shortfall of these methods is that they may also be unsuitable for the detection of apoptosis, a process that has been implicated in sperm chromatin damage[Lemasters et al. 1998]. Early apoptotic events resulting in chromatin damage are associated with increased permeability of the cell membrane to large ions [Morrison et al. 2005]. In somatic cells, annexin-V (AV) and propidium iodide (PI) are typically used to determine cells which are apoptotic and necrotic, respectively. In normal live cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane. However, in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment. Annexin-V binds to PS and thus allows for detection of late stages of apoptosis. Our efforts concentrated on screening a large number of fluorochromes to select one which would negatively select normal sperm to avoid carry-on of a foreign molecule into the oocyte and allow for very early detection of apoptotic events. Through computational screening of fluorochrome libraries, we selected fluorochromes that were least likely to negatively affect oocyte function if carry-over events occur, are excited by the 488 nm spectral line of the argon-ion laser to avoid the damaging effect of UV, and can be used for both fluorescence microscopy and flow cytometry (FC). In the current project we used a large fluorescent organic cation, PF-1, for fluorescence-activated cell sorting (FACS) to select sperm without chromatin damage. TUNEL assay was used to evaluate the number of sperm with chromatin damage. It has previously been shown that chromatin damage and apoptosis in sperm are related; therefore, in cases of sperm chromatin damage which occurs as a result of apoptosis, our hypothesis assumed that damaged sperm can be identified and removed by sorting using very early marker of apoptosis, in pursuit of improved IVF outcomes and reduced issues with embryo development.

Results

In the sorting experiment, data from all patients were combined for final analysis. Highly specific PF-1 binding allowed for clear-cut sorting as intact sperm had signal intensity less than 10^1 whereas PF-1 positive, damaged sperm, had PF-1 signal higher than 10^2 (Fig. 1). In the subfraction positive for PF-1 and PI, 431 x 10^3 of 2,167 x 10^3 sperm (19.5%) were TUNEL positive. In the non-staining group (PF-1(-) and PI(-)) only 33 x 10^3 of 2,263 x 10^3 (1.5%) were TUNEL positive. The difference was highly statistically significant (p <0.00001). The 1.5% value on TUNEL assay is a result of background fluorescence based on analysis of TUNEL performance characteristics. Incubation of sperm with PF-1 does not seem to impair motility as even PF(+) sperm has excellent progressive motility; we have included a video clip illustrating this as a supplement.

To test that PF-1 detects small changes in the apoptotic sperm population, sperm were treated with sodium nitroprusside. Of 100,000 sperm analyzed, 0.5% before and 1.2% after induction were positive for annexin-V staining, a classic stain for apoptosis in somatic cells. In the same sample, 7.8% before and 10.7% after induction were positive for PF-1 staining. A higher number of PF-1 sperm reflects better characteristics performance in assessing early apoptotic changes in sperm using PF-1. All differences were statistically significant (Table 1).

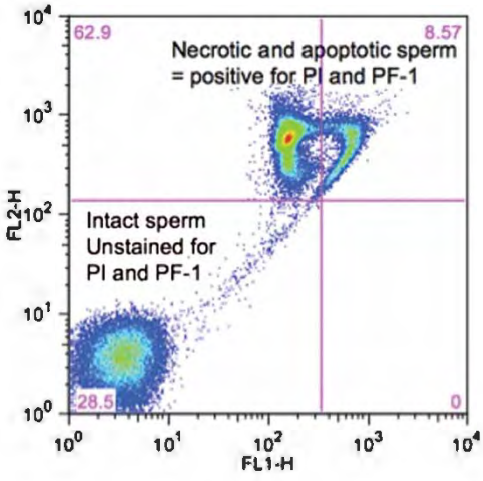

Figure 1. Bivariate plot of flow cytometry based sperm analysis following staining with propidium iodide (PI) and a proprietary fluorochrome (PF-1). The combination of two fluorochromes enables the separation of necrotic and apoptotic sperm (PI(+) and PF-1(+)) sperm from intact 'normal' sperm (PF-1(-)). Logarithmic scale transformation on the X and Y axis depicts signal intensity of PF-1 (X-axis) and PI (Y-axis). FL1-H: detection channel with 488 nm excitation and 530/30 nm filter; FL2-H: detection channel for PI.

Table 1. Change in percent of apoptotic sperm before and after apoptosis induction using annexin V and PF-1 staining.

	Non-induced		Induced		*p* value
	# of sperm (10^3)	% of total	# of sperm (10^3)	% of total	
Annexin V	471	0.5%	1,190	1.2%	<0.0001
PF-1	7,820	7.8%	10,747	10.7%	<0.0001

PF-1: proprietary fluorochrome

Mean per cent TUNEL negative and positive sperm and 95% CIs were 1.02 (0.94-1.13) and 98.6% (98.5-98.8) for negative and positive controls respectively. Distribution curves fitting showed Weibull as the most appropriate model. Normal Q-Q plot and weighted percentiles analysis revealed that assay should be rejected if negative TUNEL is > 2% ($p < 0.05$) thus indicating that any TUNEL results less than 1.9% can be considered the result of background fluorescence as in negative controls no dye was added.

The mean motility before FACS was 53.52% (SD: 10.5) and after FACS was 49.4% (SD:12.5), t-student paired test $p = 0.11$, a non-statistically significant difference. There was no statistically significant difference between in mean vitality before and after FACS: 68.8 % (SD:15.3) vs. 65.0 (SD14.99), $p = 0.33$.

Discussion

In the current study, we have demonstrated that sperm FACS with PF-1 enables sensitive and early detection of sperm with chromatin damage while preserving PF-1 negative sperm for potential clinical use. PF-1 allows for binary selection of normal and abnormal sperm as shown by distinct clustering of intact PF-1 negative and damaged PF-1 positive sperm populations during FACS (Fig.1). A significant portion (19.5%) of the group of sperm positive for PF-1 and PI staining were also TUNEL positive. This is intuitive as PF-1 detects early changes in cell membrane occurring during the beginning of apoptosis and TUNEL detects a late event, i.e., breaks in DNA. As many processes underlying chromatin damage are gradual, it takes time for DNA breaks to occur; thus one would assume that not all sperm positive for PF-1 would be TUNEL positive immediately after sorting. However this is a desirable property of our test to detect all TUNEL positive sperm and early apoptotic events to then select the best sperm. In the PF-1 negative group, only 1.5% of sperm were TUNEL positive, which is consistent with background fluorescence based on analysis of negative and positive controls for TUNEL assay. Thus PF-1 based sperm sorting selects most if not all TUNEL positive sperm and our data suggests that this method of sorting provides an efficient and effective means of separating alive and intact normal sperm from those with abnormal chromatin. The experiments with apoptosis induction using nitroprusside sodium confirm that PF-1 detects changes in membrane during early apoptosis as compared to A-V based analysis (Table 1). This can be easily explained by physicochemical differences in a way each of the fluorochrome detects apoptosis. Annexin V is a relatively late marker of apoptosis, as binding of A-V to cell membrane occurs only after cell membrane starts to disintegrate, exposing phosphatidylserine (PS) located on the cytoplasmic (inner) surface of the cell membrane. In apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment and detection by A-V. Sperm selection based on A-V would still leave a significant number of sperm which although A-V negative are apoptotic. Thus sperm sorting based on A-V would be much less clinically useful. PF-1 is sensitive and an early marker of apoptosis as it reflects subtle changes of ionic membrane potentials rather than loss of cell membrane integrity. Using PF-1 for sperm sorting we are assured that even sperm with early apoptotic events can be detected and removed from the sample.

Because the test employs large molecules that require large pores in cell membrane to enter the cell, there is little risk for residual fluorochrome to be carried-over in the isolated normal sperm. Therefore this assay may be employed as a new treatment modality for couples with male factor infertility secondary to sperm chromatin damage. This technology is significant and revolutionary in that it allows for the separation and preservation of sperm that have not undergone apoptosis and sustained chromatin damage, rather than merely identifying sperm with chromatin damage as a likely culprit for male factor infertility.

It needs to be determined to what degree selection of PF-1 negative sperm improves outcomes of IVF like fertilization, early embryo development, and pregnancy rates. In the event that sorting for chromatin damage could significantly decrease the failure rate associated with IVF and ICSI, then this technology may prove instrumental in eliciting better outcomes of ART. Given the costs and health risks that can be associated with repeated ART procedures, PF-1 based sorting has the potential to mitigate these factors in its promise to assist embryologists by providing a better tool for sperm selection than currently available.

It is our hope that if further developed, PF-1 sorting would complement current techniques that are used in the selection of optimal spermatozoa. There are numerous techniques currently employed in ART, including swim-up and density gradient centrifugation methods, as well as magnetic-activated cell sorting. While these techniques begin to provide a method of separating and grading sperm in a systematic fashion, they have deficiencies in their ability to screen for sperm on the basis of DNA integrity. The swim-up technique is dependent on the intrinsic motility of sperm, and can lose efficacy in severely asthenozoospermic samples [Aitken et al. 2011]. Swim up techniques also

pose the risk of subjecting the sperm to oxidative stress, with exposure to free radical generating leukocytes [Baker et al. 1996]. Density gradients perform poorly in situations where there are few sperm in the sample or where they are poorly liquefied [Mortimer 1994]. While these techniques do not directly evaluate for DNA damage, recent studies have shown that most techniques selecting better sperm will also reduce the number of sperm with DNA damage; this is not surprising considering that DNA damage correlates with other parameters of sperm quality like motility [Jayaraman et al. 2012]. Electrophoretically isolated spermatozoa have been demonstrated to have reduced DNA damage following evaluation by TUNEL assay [Aitken et al. 2011] however the effects of electrophysiological fields on ionic properties of sperm is yet to be explored.

Prerequisites of new, clinically relevant, and non-consumptive techniques are demanding. They must be non-invasive, accurate in discerning between healthy and damaged sperm, and also easy and expedient to perform in the clinic [Henkel 2012]. Although our work has focused on sperm DNA damage it is quite possible that other yet poorly understood biological properties of sperm may be as critical in selecting optimal sperm. Existing means of evaluating sperm in a non-consumptive manner include the utilization of cumulus cells and zonae pellucida of immature oocytes to improve selection of optimal sperm [Black et al. 2010; Franken and Bastiaan 2009; Paes Almeida Ferreira de Braga et al. 2009]. Other studies have considered the use of Raman microspectrometry; however, the technique has not been thoroughly evaluated in assessing if it improves IVF outcomes [Huser et al. 2009]. It is possible that the future will encompass multistep non-consumptive sperm selection to achieve optimal outcomes based on initial evaluation of the infertile couple [Henkel 2012]. In so far as the outlined requisites are concerned, PF-1 based sperm sorting could prove extremely useful in assisted reproductive therapy in the clinic as it dramatically reduces one of the risks of IVF failure, i.e., sperm DNA damage. It will be essential to verify, beyond the efficacy of this sorting method in the context of IVF and ART, that there is indeed total absence of fluorochrome carry-over in sperm which are selected for IVF through this method, and that there are no other disagreeable, prohibitive, or unforeseen drawbacks. We plan to continue our work using animal models and hopefully humans to help couples with recurrent IVF failures when a high degree of sperm DNA damage in ejaculated sperm has been identified.

Materials and Methods

Overview

Spent semen samples from 18 men from an andrology laboratory were cell-sorted based on PF-1 activation into PF-1 positive and PF-1 negative samples. The use of the spent sperm samples for this study was approved by the Weill Cornell Medical College Institutional Review Board. Because otherwise to be discarded and de-identified samples were used in the study, a waiver to obtain informed consent from patients was obtained. Subsequently the number of sperm with DNA damage in each fraction was measured by TUNEL assay. Sperm sorting was performed on day of semen collection.

Preparation of semen specimens

The discarded portion of the semen analysis specimen provided at a new patient visit at an infertility practice was first purified using a Percoll gradient adapted from the WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction (WHO 2010). Percoll gradient was used to resemble the most likely future clinical scenario of using PF-1 sorting when the best sperm would be first selected for ART using swim-up or Percoll gradient followed by PF-1 sorting. Once washed, the sperm concentration was estimated by viewing 4 μL of the specimen under 400x magnification and counting the number of sperm in one field.

Induction of apoptosis

As a positive control, half of each patient specimen was incubated for one h in 10^{-4} M sodium nitroprusside solution to induce apoptosis. The induction protocol was adapted from Wu and colleagues [Wu et al. 2004].

Fluorochromes and sample incubation

Semen samples of seven men were used to optimize assay conditions for the fluorescent dyes PI, A-V, and a proprietary fluorochrome (PF-1). Dead sperm are permeable to PI, dead and apoptotic cells are permeable to PF-1. Intact cells are impermeable to both. A-V binds to phosphatidylserine (PS) on the inner side of cell membranes of apoptotic cells,both live cells undergoing apoptosis and dead cells having undergone apoptosis. Fluorochromes were obtained from Invitrogen (Carlsbad, CA, USA). Sperm were suspended in PBS 1X using 12x75 mm tubes and placed on ice. Initially 1 μL of fluorochrome was added to each sample containing about 1 x 10^6 cells per mL as suggested in the manufacturer's instructions for Jurkat cells. Sperm were incubated for 30 min on ice prior to sorting. Immediately after sorting the motility and vitality were measured and 5 slides for TUNEL assay from PF-1 positive and PF-1 negative populations were prepared. We optimized the staining conditions for sperm for each fluorochrome to achieve best signal intensity. Subsequent analysis and sorting was performed using 100,000 sperm per mL. In addition 5,000 and 50,000 sperm per mL were analyzed to detect limits of detection. Overall close to 200 runs and analyses was performed during this study to optimize the sperm concentration, gating, and compensation parameters, in addition to concentration and incubation parameters of fluorochromes. Forward and side scatter were used as initial gate setting to select all sperm. Considering the non-circular shape of sperm head with tail and mid-piece adding their own signal-noise, as well as the fact that most analytical algorithms use round large cell models, it is critical to establish reliable gate parameters on each of the instruments. This need is especially common with the loss of

operator-dependent flexibility in the control of fluidics that accompanies the advancement of instruments. A polysterene microspheres panel was used to assist with size calibration and 6 μm spheres used in fluidics calibration (BD Calibrite 3 Beads, BD Biosciences, San Jose, CA, USA). As instruments differ in their sensitivity, design, and software we recommend using 1 μL of stock fluorchrome per 1 x 10^6 sperm suspended in 1 ml of PBS as initial settings to optimize the assays in other laboratories. Once assays are optimized on a specific instrument then sperm concentration can be lowered to 50,000 to 100,000 per mL. Concentration adjustment will depend on the number of TUNEL positive sperms in ejaculated samples. In men with a high number of sperm with DNA damage higher concentrations for each run are needed to achieve an adequate number of PF-1 negative (normal) sperm after sorting.

FACS

Prepared semen samples from five patients were sorted with the BD FACSVantage (BD Biosciences). Using predefined settings 488 nm excitation with green fluorescence emission and 530/30 band-pass filters were used for PF-1 based sorting. Normal spermatozoa were separated from necrotic and apoptotic cells based on PF-1 signal. To verify that proposed protocol allows for selection of intact sperm, the sorted and unsorted populations were examined microscopically for motility and viability using WHO laboratory manual 3rd edition protocol (WHO 2010). Ability of PF-1 to discriminate between DNA damaged and undamaged sperm was analyzed using TUNEL assay following a modified manufactured protocol (Roche Applied Sciences, Indianapolis, IN, USA). The percentage of TUNEL positive sperm between PF-1 positive and negative populations were compared using the chi-square test for difference in the percentage of TUNEL-positive cells.

TUNEL assay

TUNEL assay was performed using 10 μL sperm spread on each of 5 microscope slides. Two slides for negative and 2 for positive controls were analyzed and 200 sperm per slides were counted. All positive controls were incubated with 500 μL of DNAse type I (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at 37°C incubator prior to adding enzyme solution and labeling solution. For negative controls only labeling solution was added. All slides were then incubated for 1 h at 37°C, counter stained with DAPI, and analyzed by the same observer. Results were reported as percentage of TUNEL positive (FITC) sperm per total number of sperm (DAPI). Performance characteristics of TUNEL to assess low limit of detection were calculated based on 125 positive and 126 negative assays scored between April of 2009 and 2012 by the same observer (AB).

Induced apoptosis and sperm chromatin damage

Semen samples of 12 men were analyzed (7 to optimize assay conditions, 5 for experimental assays) with the fluorescent dyes PI, PF-1, and A-V. Samples were analyzed with the BD LSR II flow cytometer (BD Biosciences). Half of each specimen was incubated with sodium nitroprusside for induction of apoptosis. The populations of live, apoptotic, and dead cells with and without apoptosis-induction were compared using multicolor analysis with all 3 fluorochromes.

Statistical analysis

Statistical significance of PF-1 triggered FACS in reducing number of TUNEL positive sperm was determined using the chi-square test (JMP 9 for Mac, SAS Institute, Cary, NC, USA). Difference in motility and vitality before and after flow cytometry was analyzed using t-student paired test for difference in mean assuming equal variance distribution. (Prizm 5 for Mac, GraphPad Software, Inc, La Jolla, CA, USA). Performance characteristics of TUNEL assay were measured using JMP 9 for Mac.

Declaration of interest: Cornell University has filed a U.S. patent application on technology invented by Dr. Paduch that is discussed in this paper. The remaining authors have nothing further to disclose and report no conflicts of interest. This study was partially supported by The Frederick J. and Theresa Dow Wallace Fund of the New York Community Trust and Irena and Howard Laks.

Author Contributions: Conceived and designed the experiments: DAP, HK; Performed the experiments: HK, SM, AB, DAP; Analyzed the data: DAP, HK, MGF; Contributed reagents/ materials/ analysis tools: MG, PS, AB, SM; Wrote the manuscript: MGF, PNS, DAP.

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Adverse effect of paroxetine on sperm

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Objective: To assess the effects of a selective serotonin reuptake inhibitor on semen parameters.
Design: Prospective study.
Setting: Academic medical center.
Patient(s): Thirty-five healthy male volunteers, 18 65 years old.
Intervention(s): Paroxetine administration for 5 weeks.
Main Outcome Measure(s): Serum hormone levels, semen analyses, percent sperm DNA fragmentation, and questionnaire assessment of sexual function assessed before, during, and 1 month after drug administration.
Result(s): Mean sperm DNA fragmentation was significantly higher for men while on paroxetine (30.3%) versus baseline (13.8%). Before paroxetine, 9.7% of patients had a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) score ≥30% compared with 50% at week 4 of treatment. The odds ratio (OR) of having abnormal DNA fragmentation while taking paroxetine was 9.33 (95% confidence interval, 2.3 37.9]. Multivariate logistic regression correcting for age and body mass index confirmed this correlation (OR, 11.12). Up to 35% of men noted significant changes in erectile function and up to 47% of men reported ejaculatory difficulties on medication. Recovery to near-normal sexual function was noted after stopping treatment. Standard semen parameters were not significantly altered during paroxetine treatment.
Conclusion(s): In men with normal semen parameters, paroxetine induced abnormal sperm DNA fragmentation in a significant proportion of subjects, without a measurable effect on semen parameters. The fertility potential of a substantial number of men on paroxetine may be adversely affected by these changes in sperm DNA integrity. (Fertil Steril® 2010;94:1021 6. ©2010 by American Society for Reproductive Medicine.)

Key Words: Male infertility, semen analysis, antidepressants, sperm, DNA fragmentation, erectile dysfunction, ejaculation, body mass index

Major depressive disorders affect approximately 10% of American men over their lifetimes (1). Antidepressant medications are the most common form of treatment, with almost 233 million prescriptions written in 2007 (2). Newer agents such as selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors with equivalent inhibitory action on serotonin reuptake have supplanted older treatment options because of the perceived favorable safety and side effect profiles associated with the newer antidepressants.

Although the majority of antidepressants are prescribed for treatment of depression, they may also be used for treatment of anxiety disorders such as generalized anxiety disorder and obsessive-compulsive disorder. Antidepressant dispensing rates have continued to increase in recent years (3).

Received January 13, 2009; revised and accepted April 21, 2009; published online June 10, 2009.
C.T. has nothing to disclose. A.S.F. has nothing to disclose. M.A. has nothing to disclose. D.A.P. has nothing to disclose. P.N.S. is a member of the Medical Advisory Board for Theralogix, Rockville, MD.
Supported by the Frederick J. and Theresa Dow Wallace Fund of the New York Community Trust and the Brady Urology Foundation.
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Despite the rising use of prescription antidepressants and the known effects of SSRIs on emission and ejaculation (4), few reports have evaluated the effect of antidepressants on male fertility or sperm quality (5). In 2007, we reported two cases of men referred for male infertility evaluation who appeared to have antidepressant medication-associated changes in sperm concentration and motility (6). Both men showed marked improvements in total motile sperm counts within a few weeks after discontinuation of antidepressant medication. This rapid recovery to normal semen parameters suggested to us that SSRIs affect sperm transport, not sperm production which would take months to recover. Given that SSRIs adversely affect emission and ejaculation, it is possible that they could negatively influence sperm transport, as well, with a resultant negative impact on sperm quality and number.

Attempts to assess sperm DNA integrity as determined by sperm DNA fragmentation indices have increasingly been incorporated as part of a male fertility evaluation, although clinical indications for these tests have yet to be defined (7). DNA damage may exist independent of standard semen parameters (8) and the degree of DNA fragmentation correlates with poorer fertility and pregnancy outcomes, even when techniques such as in vitro fertilization and intracytoplasmic sperm injection are applied (9, 10).

We designed the study described herein to assess the potential impact of one SSRI, paroxetine, on standard semen parameters, sperm DNA integrity, endocrine profiles, and sexual function in healthy men. We hypothesize that SSRIs produce a negative impact on semen parameters by exerting an influence on sperm transport, not by disturbing spermatogenesis. An increase in sperm DNA fragmentation that occurs with delayed sperm transport has been observed in men with ejaculatory defects as well as men with obstructive azoospermia (11). Paroxetine was selected for use in this study because it has a relatively short half-life and has been shown previously to exert the strongest effect in delaying ejaculation (12, 13).

MATERIALS AND METHODS

Participants

Normal, healthy male volunteers (18–65 years old) were recruited to identify men with normal semen parameters and physical examinations. Exclusion criteria included: known sexual dysfunction, tobacco use, illicit drug use, alcohol intake greater than 2 ounces daily, prescription medications, history of psychiatric disorder, previous chemotherapy or radiation, history of seizure disorder, clinically detected varicocele, oligoasthenospermia or azoospermia or ongoing attempts to initiate pregnancy. Volunteers were excluded if suspicion of an Axis I psychiatric disorder was found on Structured Clinical Interview for the Diagnostic and Statistical Manual of Mental Disorders IV (14). Brief sexual function inventory (BSFI) (15), and a screening semen analysis were done at the outset. A total of 35 volunteers were enrolled after the initial screening process. All participants granted written informed consent before enrollment in the study and initiation of testing.

Study Design

A second semen sample before medication initiation was obtained at least 3 weeks later, and semen parameter results were averaged together as baseline values for each patient. Hormonal parameters were drawn between 8:00 and 10:30 AM. Specimens were obtained before starting medication to assess baseline T, FSH, LH, E_2, and PRL levels. Interassay and intraassay coefficients of variation for all hormones tested ranged from 4.4–11.6%.

Paroxetine was administered for 5 weeks using an escalating dosing schedule: week 1, 10 mg daily; week 2, 20 mg daily; weeks 3 and 4, 30 mg daily; week 5, 20 mg daily. Semen analyses were performed at the end of weeks 2 and 4. Serum blood samples and the BSFI were repeated at the end of week 4. One month after cessation of medication, each subject provided a final ejaculated specimen and again completed the BSFI. Sperm DNA integrity (deoxyuride-5′-triphosphate biotin nick end labeling [TUNEL]) assays were performed on one baseline semen sample and on the week 4 sample. Subjects were instructed to abstain from ejaculation for 2–5 days before semen analysis.

This research protocol was approved and monitored by the Weill Cornell Medical College Institutional Review Board and entered into the National Institutes of Health clinical trials database before initiation of the study.

Laboratory Testing

All laboratory personnel were blinded to samples.

Semen analyses Semen samples were collected into a sterile container and allowed to liquefy at 37°C for 30 minutes. A single technician in a New York State–certified laboratory assessed standard semen parameters using World Health Organization standards (16).

Hormonal evaluation Approximately 8 mL of peripheral blood was obtained via venipuncture and serum was separated immediately by centrifugation. Serum samples were stored at –20°C, and all samples were run in duplicates using commercially available enzyme immunoassay kits for T (TE080S) and E_2 (ES071S) and enzyme-linked immunosorbent assay kits for PRL (PR063F), FSH (FS046F), and LH (LH049F; Calbiotech, Inc., Spring Valley, CA).

Sperm DNA integrity analysis For TUNEL assays of sperm, four smears from each semen sample were prepared on glass slides and air-dried. The In Situ Cell Death Detection Kit with Fluorescein isothiocyanate (FITC; Roche Diagnostics GmbH, Mannheim, Germany) was used with modifications. Each slide was fixed with 4% paraformaldehyde (1 mL) in phosphate-buffered saline (PBS) solution and incubated at room temperature for 1 hour. Slides were washed with ice-cold PBS then permeabilized with TritonX in 0.1% sodium citrate for 5 minutes. Slides were again washed with PBS then incubated with a mixture of the TUNEL enzyme solution containing terminal deoxynucleotidyl transferase plus TUNEL labeling solution containing deoxyuridine triphosphate. A Parafilm M strip (Alcan Packaging, Darien, CT) was applied to each slide, and the slides were incubated in a dark, moist chamber at 37°C for 1 hour. After labeling, slides were taken out of the chamber, the Parafilm M was removed, and the cells were washed with PBS. Vectashield (Vector Laboratories, Burlingame, CA) with 4',6-diamidino-2-phenylindole (DAPI) was applied to each slide for DNA counterstaining, and a cover slip was applied. Cells were allowed to stain overnight. Two negative and two positive controls were tested with each batch.

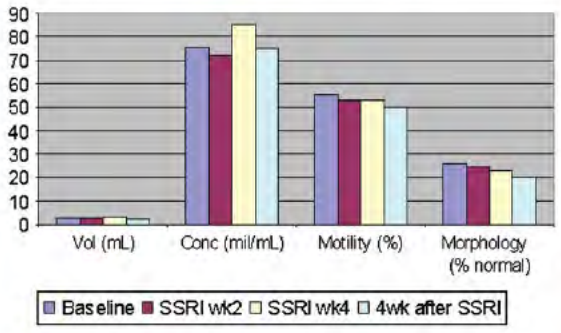
Slides were analyzed using an epifluorescent microscope at ×400 magnification. The number of DAPI-positive cells were counted then, in the same field, the number of FITC-positive cells were recorded. At least 100 DAPI-positive cells were counted for one single tally. The number of FITC-positive cells detected were divided by DAPI-positive cells × 100 to produce the percentage of TUNEL-positive cells (containing fragmented DNA), and at least four separate fields were analyzed.

Statistical Analysis

A prospective power calculation assuming a normal distribution of semen analyses with an estimated standard deviation

FIGURE 1

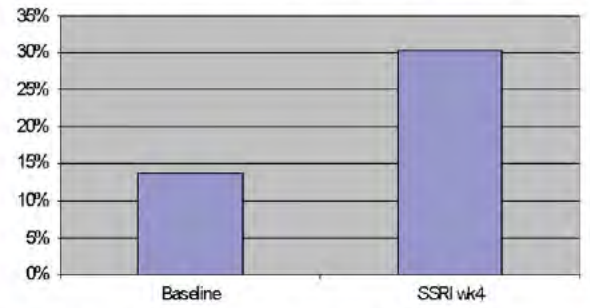
Semen parameters. Mean values for seminal volume, sperm concentration, percent total motility of sperm, and normal morphology are compared at baseline, week 2 of paroxetine, week 4 of paroxetine, and 1 month after discontinuation of paroxetine. There were no statistically significant differences (ANOVA).



Tanrikut. Effect of paroxetine on sperm. Fertil Steril 2010.

FIGURE 2

Comparison of mean TUNEL scores. Mean percentage of sperm DNA fragmentation at baseline was 13.8%; this rose to 30.3% at week 4 of paroxetine administration ($P < 0.001$, t test).



Tanrikut. Effect of paroxetine on sperm. Fertil Steril 2010.

of 25%, a 20% change in semen parameters when on medication, a two-sided alpha of 0.05, and a beta level of 0.15 yielded a required sample size of 31 subjects. Semen parameters, hormone levels, TUNEL assays, and sexual function scores for each individual were compared at each time point. Continuous data were assessed for normality using the Shapiro-Wilk test. The central limit theorem was invoked for those distributions that approached normality. Continuous data were analyzed using ANOVA and repeated measures t test comparisons for parametric data and Wilcoxon and Kruskal-Wallis tests for nonparametric data. Dichotomous measures using standardized cutoffs were evaluated by contingency table analysis. All statistical analyses were performed using SAS JMP 7.0 software (SAS Institute, Inc., Cary, NC).

RESULTS

Eighty-four men were screened, and 35 subjects enrolled. Mean age of subjects was 33.9 ± 11.1 years (range, 19–58 years) and mean body mass index (BMI) was 26.9 ± 4.4 (range, 19.4–38.1). Eleven (31.4%) patients had a BMI <25, 15 (42.9%) patients had a BMI of 25–30, and 9 (25.7%) patients had a BMI ≥30. Two patients left the study after medication initiation: one because of medication side effects and one was lost to follow-up after completing medication.

Endocrine Effects

Statistically significant decreases in serum T (844 ng/dL vs. 605 ng/dL; P 0.015, t test) and E_2 (28.8 pg/mL vs. 20.6 pg/mL; P 0.019, t test) were noted with paroxetine. However, the decreased values on medication remained well within the normal reference range for each of these hormones. There were no significant changes in serum concentrations of FSH, LH, or PRL during paroxetine treatment.

Semen Parameters

Semen parameters (volume, concentration, motility, and morphology) were not significantly altered during SSRI treatment (Fig. 1).

DNA Fragmentation

Mean TUNEL score was significantly higher on SSRI (30.3%) vs. baseline (13.8%; $P < 0.001$, t test; Fig. 2). At baseline, 9.7% of patients had a TUNEL score ≥30%, compared with 50% patients with a TUNEL score ≥30% at week 4 of SSRI administration (P 0.001, Fisher's Exact; Fig. 3). The odds ratio (OR) of having abnormal DNA fragmentation while taking an SSRI was 9.33 (95% confidence interval [CI], 2.3–37.9]. Multivariate logistic regression, correcting for age and BMI, confirmed that SSRI treatment was significantly correlated with increased DNA fragmentation (OR, 11.12; $P < 0.001$).

A subset analysis of men who had a T level decrease ≥150 ng/dL was performed to assess whether this decrease correlated with an increase in DNA fragmentation to ≥30%. No correlation was found (P 1, Fisher's exact).

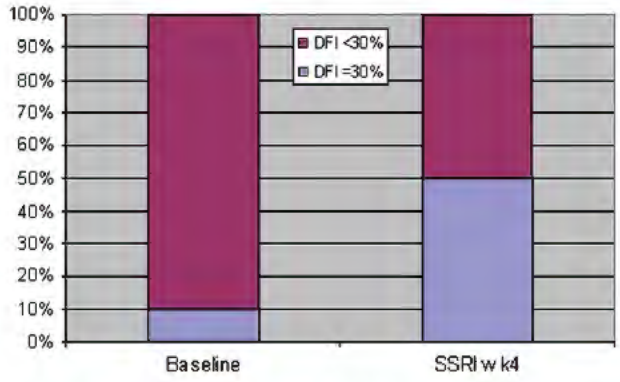
An incidental finding was that men with abnormal TUNEL score had a higher BMI. Analysis of variance revealed a mean BMI of 25.7 for TUNEL score <30% and a mean BMI of 28.2 for TUNEL score ≥30% ($P < 0.02$). A similar trend was noted when BMI and TUNEL scores were compared at baseline and on paroxetine (P 0.05 and P 0.13, respectively), but it did not reach statistical significance.

Sexual Dysfunction

The BSFI results revealed significant sexual dysfunction while taking an SSRI compared with baseline. Four questions

FIGURE 3

TUNEL results with 30% threshold. Only 9.7% of patients had a TUNEL score ≥30% before medication compared with 50% of patients at week 4 of paroxetine (P 0.001, χ-square).

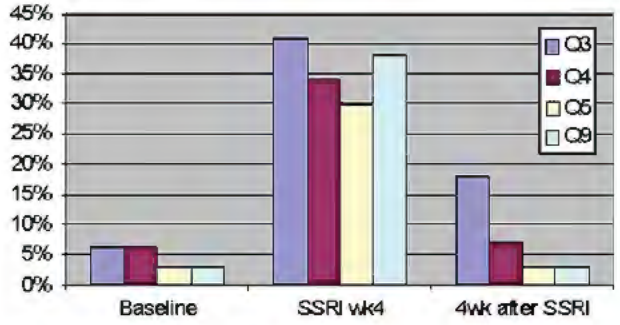


Tanrikut. Effect of paroxetine on sperm. Fertil Steril 2010.

of the BSFI address erectile function and three address ejaculatory function. Up to 35% of men noted significantly worsened erectile function ($P < 0.003$; Fig. 4), and 47% of subjects reported significant declines in ejaculatory function ($P \leq 0.002$; Fig. 5). These significant changes from baseline returned to near-normal one month after treatment. Several patients experienced severe ejaculatory dysfunction while taking paroxetine and were unsuccessful in providing a semen sample after attempts on three separate days. This occurred in two men during week 2 and in four men during week 4.

FIGURE 4

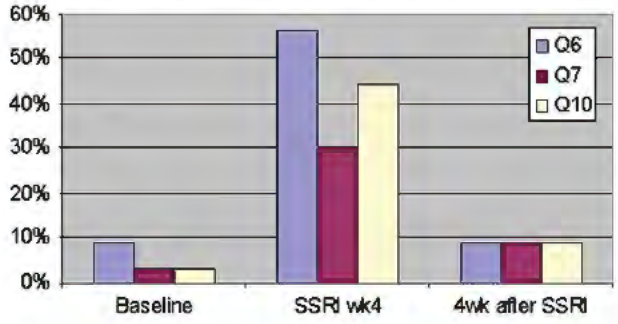
Changes in erectile function. Four questions (questions 3, 4, 5, 9) of the BSFI pertain to erectile function, rated on a scale of 0 (most severe dysfunction) to 4 (no dysfunction). The percentage of patients responding 0, 1, or 2 to the four questions increased significantly while taking paroxetine, from 3–6% to 30–41%. Erectile function returned to or approached baseline 1 month after cessation of medication ($P < 0.003$, χ-square).



Tanrikut. Effect of paroxetine on sperm. Fertil Steril 2010.

FIGURE 5

Changes in ejaculatory function. Three questions (questions 6, 7, 10) of the BSFI assess ejaculatory function, rated on a scale of 0 (most severe dysfunction) to 4 (no dysfunction). The percentage of patients responding 0, 1, or 2 to the three questions increased significantly while taking paroxetine, from 3–9% to 30–56%. Ejaculatory function approached baseline 1 month after cessation of medication with only 9% of patients complaining of more than mild dysfunction ($P \leq 0.002$, χ-square).



Tanrikut. Effect of paroxetine on sperm. Fertil Steril 2010.

DISCUSSION

This study sought to further evaluate the effect of SSRIs on male fertility potential after previously published anecdotal observations identified dramatically affected total motile sperm counts associated with SSRI use (6). In this study, we have demonstrated that marked changes in sperm DNA fragmentation occur during paroxetine treatment that are not reflected by changes in standard semen parameters. Not only did mean DNA fragmentation levels increase from 13.8 to 30.3% on paroxetine, but the percentage of patients with abnormal DNA fragmentation (≥30%) rose from 9.7–50%. Integrity of DNA is important to normal fertility (17, 18) and affects the success of intrauterine insemination. Abnormal sperm DNA integrity also affects pregnancy outcomes with the most advanced assisted reproductive technologies (10, 19, 20). The threshold of ≥30% sperm DNA fragmentation has been suggested as a cut-off point to identify men with poorer fertility (21). The fivefold increase in the number of patients who developed abnormal sperm DNA integrity while taking paroxetine in this study is unsettling. Although fertility was not directly assessed in this study, these marked changes in the DNA integrity of sperm suggest an adverse fertility effect related to paroxetine use.

Both serum T and E_2 levels decreased significantly during treatment in this study. Two other studies that have included hormonal assessment of patients who were receiving an SSRI (fluoxetine) for major depressive disorder have not shown any significant changes in serum T during treatment (22, 23). Because the lower values in our study were well within the normal range for each hormone, the differences are likely

of little clinical relevance in healthy men. However, low or low-normal serum testosterone levels are often found in men examined for a fertility evaluation. In those men, an approximately 28% decrease in serum T level levels could have clinical relevance with symptoms of hypogonadism and/or potential negative impacts on spermatogenesis. The drop in T and E_2 levels that was noted in our study may partially explain recent reports of increased fractures in older patients using SSRIs (24, 25).

Of note, changes to sperm DNA quality occurred without changes in standard semen parameters with paroxetine. For patients taking SSRIs and desiring fertility, the standard semen analysis would not show sperm DNA damage. Serotonin affects the ejaculatory response and sperm transport. The extent of this effect may vary from patient to patient. Whereas limited changes in serotonin-related effects may moderately slow sperm transport resulting only in altered sperm DNA integrity without changes in sperm numbers, a limited number of men may experience more dramatic effects on sperm transport causing deterioration of standard semen parameters, such as those patients reported in our prior publication (6). Again, the rapid recovery of semen parameters in the initial case reports coupled with the lack of change of FSH in this study supports a mechanism of impact via sperm transport rather than sperm production, although basic science studies would be warranted to confirm this concept.

Patients already taking an SSRI were not considered for this study, as we could not test for changes in semen parameters if subjects were already taking medication. Furthermore, for patients who are clinical candidates for treatment of anxiety or depression with SSRIs and have not yet started medication, their inherent psychiatric condition could affect sperm production, thereby creating confounding variables in evaluating the effects of paroxetine. As an initial investigation of our case study findings, this was a proof-of-principle study and a placebo arm was not included because of the additional significant expense that would have been incurred for the study. Future studies may incorporate a placebo-controlled design.

The incidental finding of a relationship between BMI and sperm DNA fragmentation is of interest. Although other studies have reported that increased BMI is associated with lower sperm concentration and decreased motility (26, 27), we are aware of only one other study that identifies an association between high BMI and elevated sperm DNA fragmentation (28). This relationship will be evaluated further in future studies.

The potential compromise of male fertility caused by increased sperm DNA fragmentation associated with paroxetine use is an important concern, given the prevalence of depressive disorders and the upward prescribing trends for SSRI antidepressants. It remains to be seen whether similar degrees of DNA fragmentation occur with alternative SSRIs or other classes of antidepressants. We plan future larger-scale, randomized, placebo-controlled trials with other SSRIs to further explore these findings and possibilities.

Acknowledgments: The authors thank Richard Lee, M.D., and Alex Bolyakov, M.S., who assisted with TUNEL assays and performed hormonal assays, respectively. Marc Goldstein, M.D., supervised semen analyses performed for this study, with our gratitude. We greatly appreciate the efforts of PeggyAnn King, R.N., who assisted with subject medication teaching and blood draws. All contributors are affiliated with the Cornell Reproductive Medicine Institute, Weill Medical College of Cornell University, New York, New York.

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American Journal of Medical Genetics Part C (Seminars in Medical Genetics) 163C:16–26 (2013)

ARTICLE

Endocrinological Issues and Hormonal Manipulation in Children and Men With Klinefelter Syndrome

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[1]

47, XXY or Klinefelter syndrome (KS), the most common chromosomal aberration in males, is characterized by either absolute or relative hypogonadism with frequent decline in serum testosterone (T) following the onset of puberty. Decreased T levels are the result of testicular dysfunction with decrease in size of Leydig cells, and loss of germs and Sertoli cells leading to tubular hyalinization. Increase in estradiol results from over-expression of aromatase CYP19. Deficient androgen production and observed varied response of end-organs to T leads to delayed progression of puberty with decreased facial/body hair, poor muscle development, osteoporosis, and gynecomastia. It is possible that hypogonadism and excessive estradiol production contribute to emotional and social immaturity, and specific learning disabilities in KS. Based on the authors' experience and literature review, early fertility preservation and hormonal supplementation may normalize pubertal development, prevent metabolic sequelae of hypogonadism, and have a positive effect on academic and social development. No randomized clinical trials are available studying the effects of T supplementation on reproductive or cognitive issues in KS. Aggressive T supplementation (topical gel) and selective use of aromatase inhibitors may be considered at the onset of puberty with careful follow-up and titration to reach age-specific high-normal physiologic serum values. The decision to institute hormonal therapy should be part of a multidisciplinary approach including physical, speech, behavioral, and occupational therapy.

KEY WORDS: Klinefelter syndrome; hypogonadism; testosterone surge; testosterone replacement therapy; aromatase inhibitors; nonobstructive azoospermia; fertility; meiosis; spermatogenesis; Leydig cells; Sertoli cells; germ cells

How to cite this article: Wosnitzer MS, Paduch DA. 2013. Endocrinological issues and hormonal manipulation in children and men with Klinefelter syndrome. Am J Med Genet Part C 163C:16–26.

INTRODUCTION

Klinefelter syndrome (KS) is the most common cause of male hypogonadism and chromosomal aberration occurring in 0.2% of the general population, comprising up to 4% of patients in male reproductive practices and 15% of azoospermic males [Bojesen et al., 2003; Ghorbel et al., 2012]. KS genotype is caused by meiotic nondisjunction, most commonly resulting in the 47, XXY karyotype (X disomy), with variable phenotype often indistinguishable from boys with normal karyotypes on physical examination. Men with more than two X chromosomes (48,XXXY) are more affected than men with 47,XXY karyotype [Samango-Sprouse, 2001]. Given the wide phenotypic spectrum, less than 10% of men are diagnosed before puberty and many men with KS remain undiagnosed. Variation in phenotype may be explained by hormonal and genetic background differences, including androgen receptor polymorphism in the CAG_n repeat and skewed inactivation of additional genetic material on the X chromosome [Zechner et al., 2001; Bojesen et al., 2011]. Classic KS phenotype typically includes micropenis, small, hard testes with adolescence-onset testicular failure (spermatogenic and steroidogenic), hypergonadotropic hypogonadism with low testosterone, infertility, tall

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Grant sponsor: The Frederick J. and Theresa Dow Wallace Fund of the New York Community Trust.

Grant sponsor: Robert Dow Foundation.

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DOI 10.1002/ajmc.31350

Article first published online in Wiley Online Library (wileyonlinelibrary.com): 18 January 2013

eunuchoid stature (over 50% of males exceeding the 97th centile of height for their age), sparse facial and pubic hair, atypical motor function, behavioral issues, and specific mild to moderate deficits in language based skills with decreased verbal intelligence quotient, attention, and auditory processing [Klinefelter et al., 1942; Ross et al., 2008; Zahn-Waxler et al., 2008]. KS is associated with obesity and hyperestrogenism throughout life as well as increased risk of cancer (breast/germ cell), endocrine complications (diabetes mellitus, growth hormone deficiency, hypothyroidism, hypoparathyroidism), autoimmune diseases, and decreased bone density.

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Early diagnosis of 47, XXY and proactive testosterone replacement along with multidisciplinary approach for physical, speech, behavioral, and occupational therapy promotes effective developmental, social, and academic progress [Paduch et al., 2009; Radicioni et al., 2010]. It is important to remember that most descriptions of the phenotype and associated comorbidities are derived from studies of older patients who were not treated during puberty and early adulthood.

MATERIALS AND METHODS

Systematic review was conducted of the relevant literature using the Pubmed NLM database to search for primary and review articles using keywords "Klinefelter syndrome" and "testosterone," "testosterone replacement," "aromatase inhibitors," "spermatogenesis," "Leydig cell dysfunction," "partial androgen resistance". In addition data based on 200 children, adolescents, and young adults seen at our center's specialized KS clinic was used.

RESULTS

Diagnosis of 47, XXY

Cytogenetic diagnosis is completed by karyotyping of typically 20 mitotic spreads from peripheral blood. Historically the presence of Barr body in mucosal scraping was used. More recently fluorescence in situ hybridization (FISH), or molecular techniques like methylation-specific polymerase chain reaction (PCR) based on inactivation pattern differences of genes on the X chromosome: familial mental retardation gene 1 (*FMR1*) and X chromosome inactivating transcript (*XIST*) are being used to overcome the high cost of cytogenetics and the limited sensitivity of detecting low levels of mosaicism with cytogenetics [Mehta et al., 2012]. Peripheral blood cytogenetics in children diagnosed in utero should be performed to confirm prenatal diagnosis. Y chromosome microdeletion analysis is performed in azoospermic men.

Life-Long Principles of Evaluation and Management of 47, XXY (KS) Patients

Perinatal evaluation with testosterone treatment from 3 to 6 months after birth is the accepted standard of care. Fifty milligrams of Depo-testosterone (intramuscular injection once/month) for 3 months has been used. Alternatively 1 pump of Androgel 1% (1.25 g/day; 37.5 g/month) may be used for older infants with attention to any skin reaction. Response to T treatment measured as increase in penile length is a good indicator of overall tissue sensitivity to testosterone. During the prepubertal period, focus on physical, occupational, and speech therapy is critical for normal academic progress. Between birth and 10 years of age, androgen treatment is used only for very short periods in children who did not receive the 3-month treatment after birth or if the initial response was not adequate. In our practice we limit the 3-month treatment in prepubertal boys to age 8 to avoid initiation of early puberty. During puberty, physical examination with measurement of testicular volume is performed every 6 months. All men with KS should undergo a complete hormonal profile which includes follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone, estradiol, prolactin, inhibin-B, insulin-like growth factor-1 (IGF-1), and cortisol levels every 6 months starting prior to the predicted start of puberty. Thyroid and lipid profiles are obtained once a year. When FSH and LH increase, a multidisciplinary discussion with patient and parents is completed to discuss fertility management options. Morning urine samples can be used to identify sperm, but in adolescents who ejaculate spontaneously, analysis of a semen sample may be considered following discussion with parents and patient. If sperm are identified in semen, semen cryopreservation is offered. Anastrozole 1 mg once a day is used for 6 months, and additional semen samples are obtained for cryopreservation to store at least 4 6 vials of

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ejaculated sperm. For adolescents without sperm in ejaculate, the option of testicular sperm retrieval during puberty or in adulthood should be discussed. Bone density testing (dual-energy X-ray absorptiometry, DEXA) should also be completed routinely given the risk of osteopenia and osteoporosis with low testosterone. If decreased bone density is identified, additional calcium, phosphorus, parathyroid hormone, and vitamin D3 are obtained. Despite conflicting evidence for increased breast carcinoma risk, patients are taught early regarding self-examination to identify abnormal breast nodules or nipple discharge.

Postnatal Testosterone Surge

Testosterone is secreted at adult levels during three periods of male life including transiently during the first trimester of pregnancy (male genital tract differentiation) and during early neonatal life as a perinatal androgen surge (minipuberty) between 2 and 6 months of age. The third period starts during puberty and continues throughout the male life span with varied efficiency. It is postulated that decreased testosterone production occurs during all three periods in males with KS, although there are studies both in favor and against this in the 47,XXY fetus, during infancy, and during pre-puberty [Salbenblatt et al., 1985; Ratcliffe et al., 1994; Lahlou et al., 2004; Cabrol et al., 2011]. Testosterone levels normally increase up to 10 nmol/L during the first months of life and some studies show that the spike in T production within the first 3 months is decreased in boys with KS. This may be related to the inappropriate function of fetal Leydig cells, but the reason for lower T levels in newborns with KS is not completely understood. In KS patients, confirmatory postnatal karyotype, early evaluation by a physician experienced with management of KS, and measurement of penile size and testosterone level are critical. Failure to exhibit the dramatic changes that occur during normal puberty (approximately 30-fold increase in testosterone levels) is due to testicular abnormalities affecting Leydig cell function. In most KS males, testosterone rises during puberty and subsequently plateaus [Salbenblatt et al., 1985]. However despite the increase, rate of progression and testosterone levels achieved seem to be less prominent than in non-KS adolescents.

Sexual Development

In 47, XXY (KS), testosterone levels characteristically decline during late adolescence and early adulthood. Onset of puberty in KS patients occurs at a predicable time, but decreased androgen production and hyperestrogenism results in delayed progression of puberty with decreased facial/body hair, muscle development, eunuchoid features, and gynecomastia [Smyth and Bremner, 1998]. Based on our experience it seems that men with KS have varied degrees of partial androgen resistance. This manifests as attenuated suppression of SHBG during TRT as compared to non-KS men, and poor androgenization despite high normal levels of T achieved with TRT. The pubertal growth spurt is the same as in 46,XY boys, and prepubertal males with KS have similar testosterone, LH, FSH, inhibin B, and anti-Mullerian hormone (AMH) until the initiation of puberty. Based on our observations (Paduch DA, unpublished work), penile girth may be decreased in some adolescents, and boys with 48,XXXY may have thickening of penile skin with circumference exceeding length. Scrotal development is normal, but testicular size is significantly decreased during puberty (occurs between Tanner stages II III) secondary to progressive deterioration of germinal epithelium, Sertoli cells, and peritubular fibrosis while the epididymis is spared. In addition to testicular dysfunction, some boys may also demonstrate growth hormone deficiency which further impairs muscle development and peak pubertal bone mineral density [Rossodivita and Colabucci, 1994; van den Bergh et al., 2001; Bahillo-Curieses et al., 2011].

Leydig Cell Dysfunction in Klinefelter Syndrome

Testicular degeneration in KS patients may occur from spatially and ontogenically incorrect gene expression from an additional X chromosome or failure in cell divisions. During meiosis, abnormal paring of sex chromosomes leads to meiotic arrest and subsequent germ cells apoptosis. It is fascinating to notice that most somatic cells proceed through normal mitotic divisions despite of presence of additional X chromosome. However, spermatogonial stem cells, Sertoli cells, and Leydig cells undergo varied degrees of degeneration leading to infertility and hormonal abnormalities [De Sanctis and Ciccone, 2010]. There is conflicting information about timing of degeneration with some studies describing loss of spermatogonia beginning in infancy [Mikamo et al., 1968]. Diminished spermatogonia with normal Leydig and Sertoli cells have also been reported to first occur in pre-pubertal boys with KS [Muller et al., 1995], although the majority of boys have spermatogonia identified in early adolescence [Wikstrom et al., 2007]. Patients with KS demonstrate reduced adult dark spermatogonia, with gradual loss mediated by massive apoptosis preceding hypergonadotropic hypogonadism with elevated gonadotropin levels and decreased testosterone levels [Wikstrom et al., 2004, 2007]. *TEX11*, an X-chromosome derived germ-cell-specific protein expressed in spermatgonia and spermatocytes may lead to spermatogenic defects in KS [Yu et al., 2012]. Germ cells in patients with KS are also characterized by maturational arrest occurring either at early stages with type A spermatogonia before meiotic division or during later stages [Lanfranco et al., 2004; Wikstrom et al., 2004; Sciurano et al., 2009]. Studies of INSL3 indicate that Leydig cells in male infants with nonmosaic KS are sensitive to LH and such sensitivity is not diminished during the first year of life [Cabrol et al., 2011]. However, studies which use very short periods of injections of hCG to test steroidogenic activity of Leydig cells fail to measure Leydig cell activity under pulsatile and continuous release of LH occurring from puberty forward. Therefore, results of hCG stimulation tests have to be carefully interpreted. It is clearly established that T production is

regulated by acute response to LH and chronically regulated at the level of gene transcription. Most studies show that irrespective of age, median T values in men with KS are lower; thus defective Leydig cell function or excessive conversion of T to estradiol within the testis is a paramount characteristic of KS.

Steroidogenesis is initiated by activation of the LH receptor leading to an increase in cAMP, phosphorylation of cAMP-dependent kinase and activation of steroidogenic acute regulatory protein (StAR) and peripheral-type benzodiazepine (PBR) receptor, a rate-limiting step in steroidogenesis. Cholesterol is transported by StAR and PBR to mitochondria where cholesterol is converted to pregnenolone by CYP11A1 (a precursor for steroidogenic activity in Leydig cells). Current evidence suggests that hormonal regulation in Leydig cells is also mediated by multiple signal cascades including cAMP-protein kinase A (PKA), serine/threonine AKT kinase (AKT, also called protein kinase B or PKB), phosphatidylinositol 3-kinase (PI-3K), protein kinase C (PKC), mitogen-activated protein kinases (MAPKs), and intracellular Ca^{2+} signaling proteins. In addition, other biologically active agents including growth factors, steroids, prostaglandins, and cytokines can influence Leydig cell response through endocrine, autocrine, or paracrine regulation. This is clinically important since improved understanding of steroidogenic defects in men with KS will enable identification of specific treatment options [Azhar and Reaven, 2007]. In adult human Leydig cell culture, the steroidogenic activity after LH stimulation is decreased (Fig. 1). Experiments performed in our laboratory indicate that the decrease in steroid synthesis occurs downstream from CYP11A1 regulated conversion of cholesterol to pregnenolone. Adult KS testes are characterized by tubular hyalinization with most of the testicular space occupied by Leydig cells often called "Leydig cell hyperplasia" or "hypertrophy." Based on morphometric studies performed in our lab, Leydig cell size is decreased and there is no evidence that Leydig cells undergo active cell division or that their total number is higher than in non-KS testis (Fig. 2; Paduch DA, unpublished work). The majority of Leydig cells in KS have normal morphology [Regadera et al., 1991; Aksglaede et al., 2011]. Studies of isolated Leydig cells illustrate

Abnormalities of Sertoli cells with failure to mature, and fewer androgen receptors with cytoplasmic rather than cell surface location have been demonstrated.

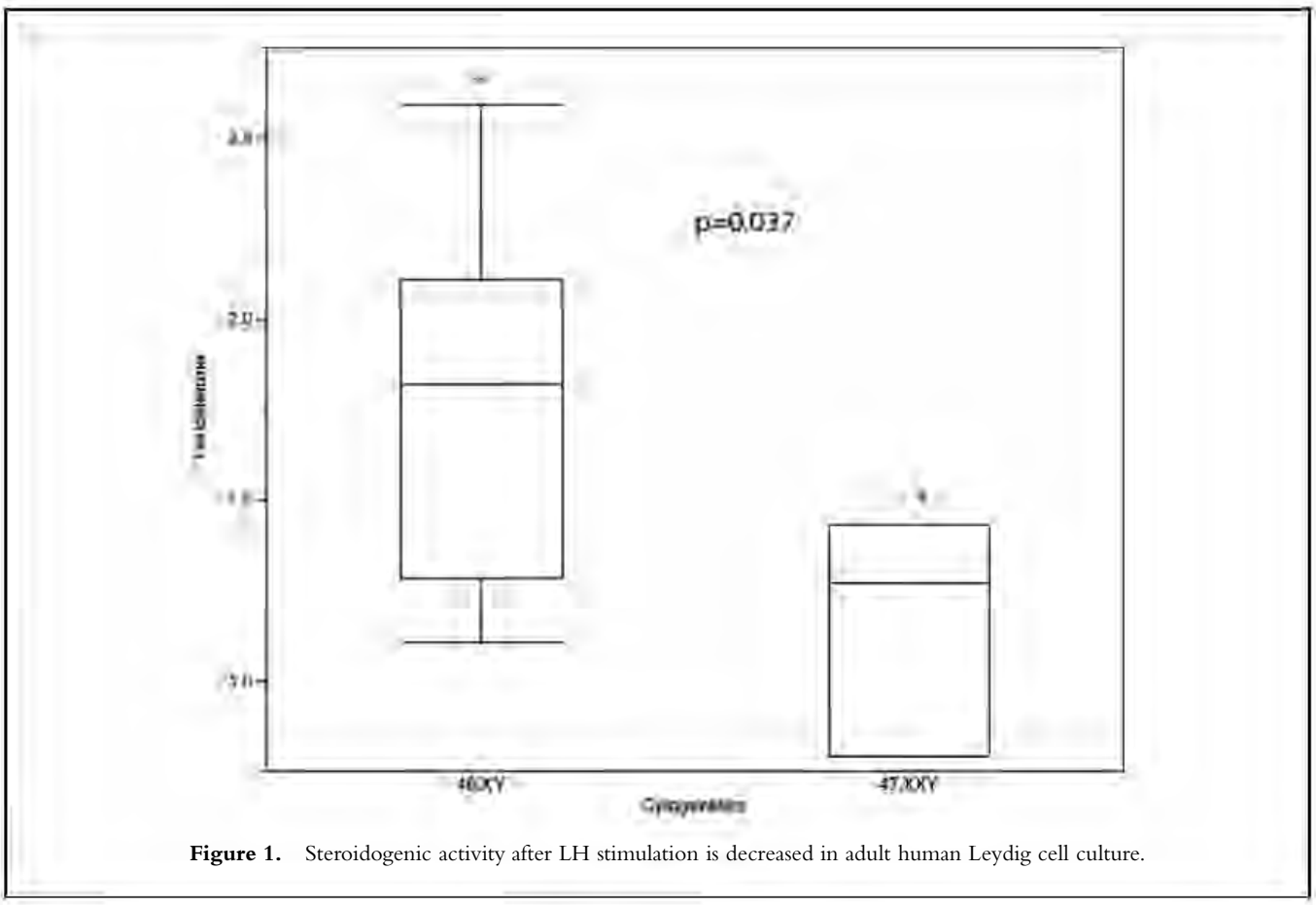

Figure 1. Steroidogenic activity after LH stimulation is decreased in adult human Leydig cell culture.

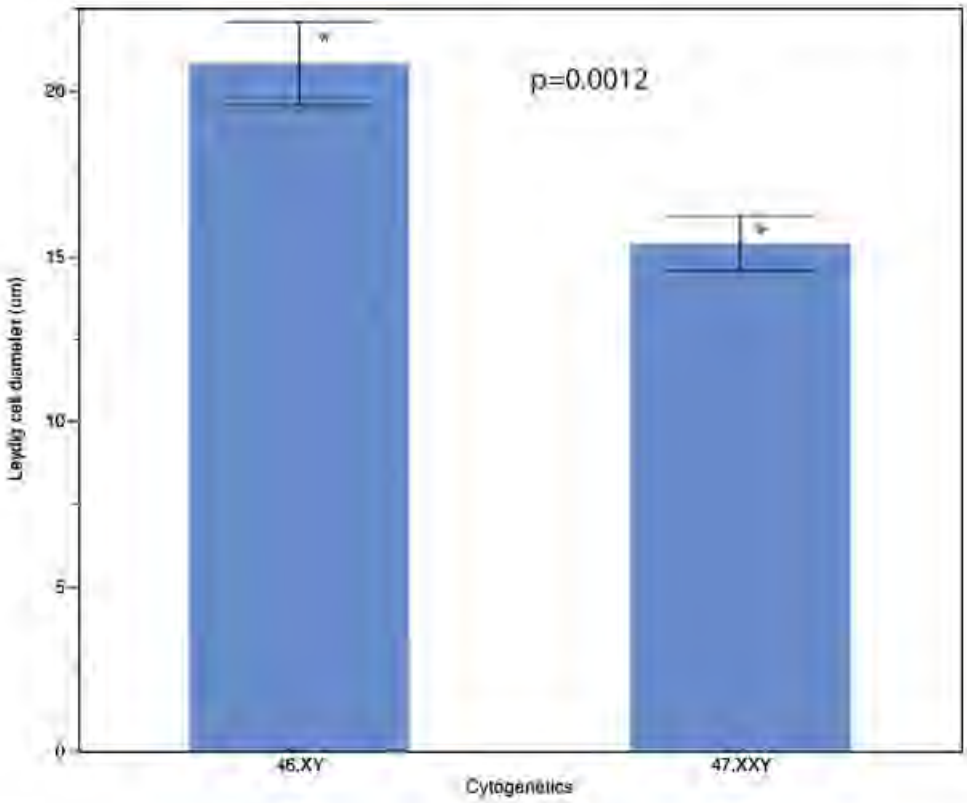

Figure 2. Morphometric analysis: Leydig cell size is decreased.

that estradiol suppresses testosterone production in up to 40%, and inhibition of estradiol by selective estrogen receptor antagonist reverses this process. Additionally our group has shown that expression of aromatase CYP19, which converts T to estradiol, is four times higher in the testes of KS males (Fig. 3). Abnormalities of Sertoli cells with failure to mature, and fewer androgen receptors with cytoplasmic rather than cell surface location have been demonstrated [Wikstrom et al., 2004, 2007]. Sertoli cell marker secretion and expression are dramatically decreased by the completion of puberty [Wikstrom et al., 2007]. Additional study is required to determine whether impaired spermatogenesis in KS results from these changes in germ cells, Sertoli cells, Leydig cells, elevated intratesticular estradiol, or due to interaction between these constituents. Small testicular size is the only consistent physical feature in 47,XXY, but the difference in size of testes between 46,XY and 47,XXY boys does not become evident until at least Tanner stage II.

KS and Infertility

Men with KS are commonly infertile because of primary testicular failure. While infertility affects 97% of KS patients, KS adolescents (Tanner stages II III) have few sperm identified in 70% of cases, and less than 10% have adequate sperm in ejaculate (cryptozoospermia or oligospermia) for cryopreservation. There is likely a specific time period in early puberty during which ejaculated sperm or sperm from testicular biopsy may be obtained for cryopreservation (Fig. 4). Although most adult men are azoospermic, rarely KS mosaic cases have had successful pregnancy without assisted medical technology [Terzoli et al., 1992]. Adolescents with KS start masturbation at the same age as 46 XY males, but have delayed ability for ejaculation (mean difference between first masturbation and first ejaculation

With the widespread use of microdissection testicular sperm extraction (TESE) and intracytoplasmic sperm injection for nonmosaic KS, patients with KS have increased chances of successful sperm recovery (≥50%) and subsequent reproduction considered equivalent to men with nonobstructive azoospermia of other causes.

2 months in 46 XY vs. 9 months in KS patients) likely secondary to relative testosterone deficiency and delayed development of the spinal cord motor generator, a sexually dimorphic S2 S4

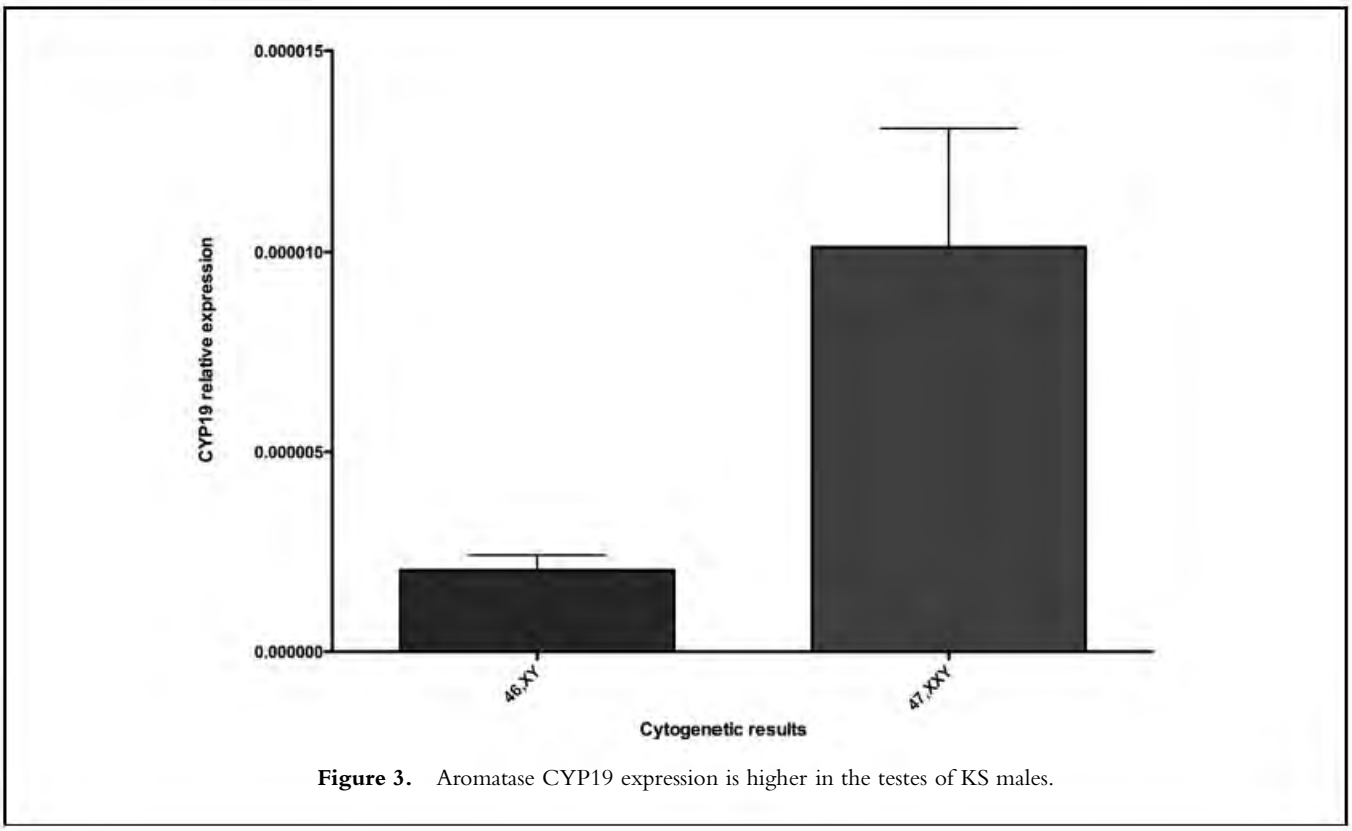

Figure 3. Aromatase CYP19 expression is higher in the testes of KS males.

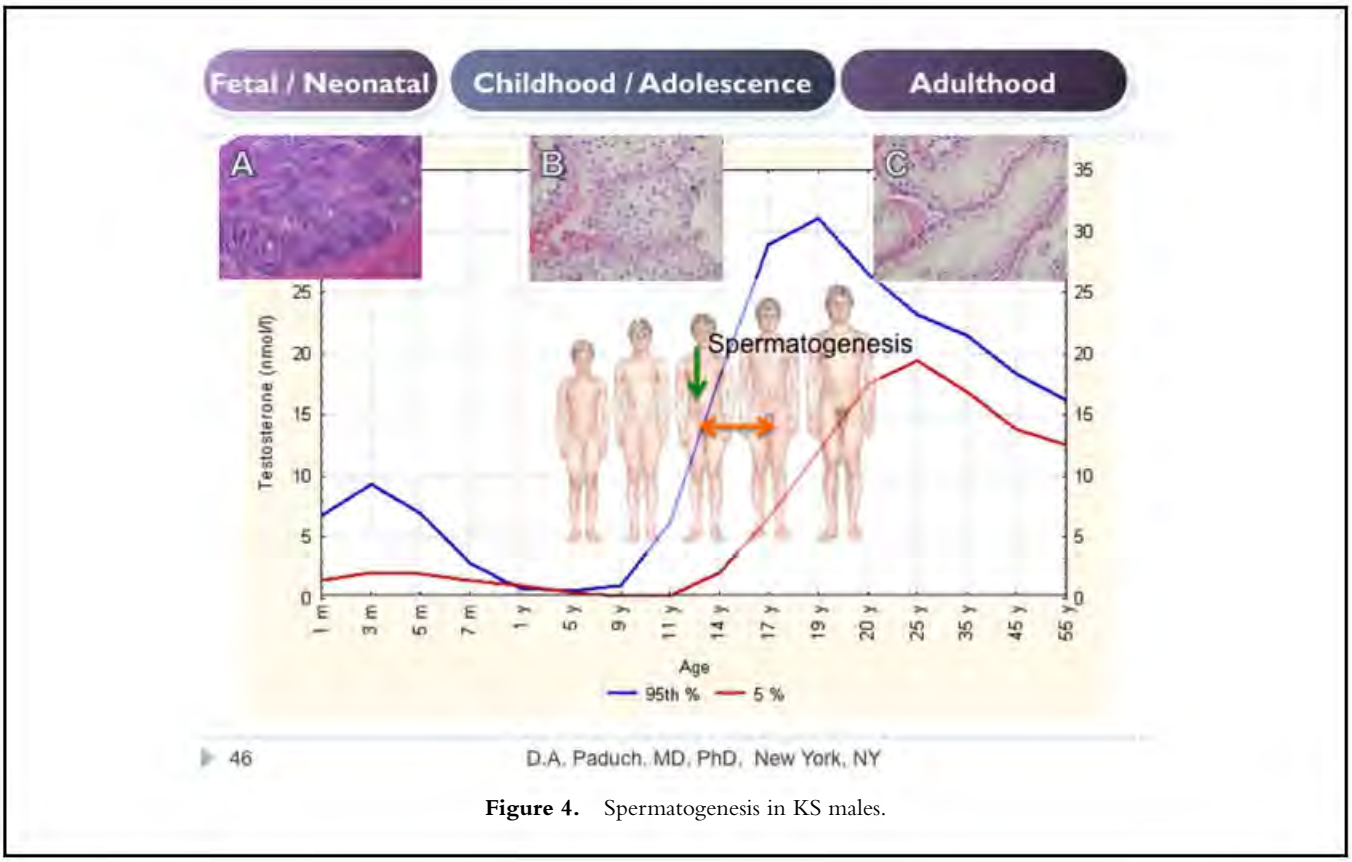

Figure 4. Spermatogenesis in KS males.

center which is dependent on testosterone. With the widespread use of microdissection testicular sperm extraction (TESE) and intracytoplasmic sperm injection for nonmosaic KS, patients with KS have increased chances of successful sperm recovery (≥50%) and subsequent reproduction considered equivalent to men with nonobstructive azoospermia of other causes [Palermo et al., 1998; Schiff et al., 2005; Ramasamy et al., 2009]. Men with normal baseline testosterone had the highest sperm retrieval rate (86%), while those requiring medical therapy who responded with a testosterone of 250 ng/dl or greater had increased sperm retrieval rate (77%) than those who had less optimal testosterone response (55%). This finding indicates that optimal spermatogenesis requires an optimal intratesticular hormonal environment. Serum FSH at baseline did not affect sperm retrieval. Successful TESE has been completed with cryopreservation of rare sperm in 75% of adolescents (Paduch, DA, unpublished work). While some groups recommend more conservative approaches, our group recommends discussion of microsurgical testicular sperm extraction and cryopreservation during puberty and young adulthood [Oates, 2012]. To the best of our knowledge, the effects of hormonal manipulation on sperm retrieval rates in adolescents have not been studied outside of our center. When sperm was identified, all adolescents were on testosterone supplementation using topical testosterone and most of them used anastrozole 1 mg a day for 6 months prior to retrieval. The hormonal manipulation in adolescents should result in catch-up in progression of puberty, normal strength, agility, muscle mass, and bone mineral density a goal which can be achieved in most adolescents if treated early. The improvements in academic and social development noted in boys with KS treated with T may be a direct result of normalization of hormonal profile on brain development or secondary to increased attention to academic progress, gain in self-confidence, and selection bias. These three goals of normal academic progress, age adequate psychosocial development, and physical maturity take priority over any effects of hormonal manipulation on fertility. It is critical to recognize that there is a significant difference in FSH and LH suppression between injectable testosterone and topical testosterone. Injectable testosterone suppresses FSH and LH and therapy with injectable testosterone must be stopped prior to any infertility treatment. Topical testosterone, however, may be used as long as there is no significant suppression of FSH and LH. The incorporation of nontestosterone hormonal therapy (clomiphene citrate, aromatase inhibitors, hCG) may be beneficial in circumstances requiring increased testosterone prior to sperm retrieval. The impact of testicular dissection for sperm extraction has been identified to result in serum T decline with recovery in 12 18 months postoperatively, with rare testicular atrophy or persistent hypogonadism [Ramasamy et al., 2005; Takada et al., 2008].

Role and Effects of TRT and Aromatase Inhibitors

Testosterone supplementation in patients with 47, XXY (KS) promotes male phenotype development, increasing penile size, decreasing gynecomastia, abdominal obesity, and improving cognition [Ruvalcaba, 1989; Bojesen et al., 2010]. There are isolated reports of androgen therapy during infancy based on androgen therapy for micropenis data, but there is not enough data to draw significant conclusions. Samango-Sprouse (personal communication, 2012) report on significant increases in cognitive and speech and language development at 36 and 72 months in a cohort of infants with 47, XXY (KS) who were treated with testosterone ethanate once a month for 3 months. Randomized clinical trial data of TRT in patients with 47, XXY (KS) are not available [Mehta and Paduch, 2012]. Options for TRT for patients with 47 XXY (KS) include initiation early to mid puberty, or at the start of symptomatic hypogonadism [Lanfranco et al., 2004; Bojesen and Gravholt, 2007; Wikstrom and Dunkel, 2011]. While specific guidelines do not exist for TRT in KS patients, age-specific testosterone formulations and dosages may be obtained from the Endocrine Society's Clinical Practice Guidelines [Bhasin et al., 2010]. We advocate implementation starting at puberty (approximately age 11 years) with titration to maintain appropriate physiologic serum testosterone levels, gonadotropins (LH, FSH) and estradiol throughout puberty [Winter, 1990]. Such a regimen ensures normal completion of puberty and prevents well-established consequences of androgen deficiency. Males with KS may require increased doses of topical testosterone secondary to partial androgen resistance (see below). Topical

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testosterone is the preferable route of administration since physiologic levels of testosterone are achieved without complete suppression of LH and FSH (unlike injectable testosterone which is also painful and intimidating for children and adolescents). In pre-adolescent boys, topical testosterone (i.e., Androgel 1%, Abbott, Abbott Park, IL) is applied at one pump (1.25 g gel/day or 37.5 g/month) for 6 12 months, with progressive increase in dosing according to T levels and progression of puberty, assessed every 6 months. Pubic and axillary hairs are much more accurate measures of progression of puberty as many adolescents have poor facial hair development. Body odor, increase in penile size, nocturnal emissions, and acne are good indicators used to measure response to treatment. For adolescent males, two pumps (2.5 g gel/day) is the recommended starting topical testosterone (Androgel 1%). Once an adolescent starts using three pumps of Androgel 1% (3.75 g/day or 112 g/month) he can switch to more concentrated preparations to decrease the amount of gel applied. Patient preference and compliance are critical in selection of the specific preparation available on the market. Underarm testosterone preparations seem to be preferred in adolescents as it is similar to deodorant application hence more familiar and less stigma-prone to adolescents. We start at 30 mg of Axiron (Eli Lilly, Indianapolis, IN) and increase every 6 12 months as needed (Table I). It is important to recognize that none of the available topical testosterone preparations in the United States are FDA-approved for use in men younger than 18. The goal is to have the serum testosterone level at the upper end of the normal age-specific range. Therapy should be monitored at 6 weeks following initiation and dose-adjusted every 6 months as determined by monitoring puberty and clinical response. Because KS adolescents frequently exhibit decreased response to TRT when compared to an age-matched cohort, vigilant follow-up is required to monitor testosterone response and medication titration to reach desired goal testosterone levels.

Given the elevation of circulating estrogens associated with increased adipose and aromatase CYP19 activity in KS, an alternative to exogenous testosterone therapy includes the use of aromatase inhibitors such as anastrazole (AstraZeneca, Wilmington DE) [Schlegel, 2012]. With decreased T/E ratio, anastrazole 1 mg daily for up to 2 years is helpful in KS adolescents with gynecomastia or central obesity who are nonresponders to maximal doses of topical testosterone (Androgel 1%, 10 12 g/day). This mechanism promotes bone health, and may alleviate the suppressive effects of exogenous testosterone on the hypothalamic pituitary axis gonadotropins. Such therapy has been shown to have positive effects on intratesticular testosterone, testosterone production, and spermatogenesis in KS males [Raman and Schlegel, 2002]. Anastrazole has also been used without significant side effects in teenagers with short stature [Faglia et al., 2000]. Additional studies will be required in order to determine the ideal route and duration of TRT/aromatase inhibitors for 47, XXY (KS) patients. Prior to initiation of hormone supplementation, fertility preservation, ethical, and legal concerns should be discussed with the patient and his parents. Effects of anastrozole on cognition have been suggested by some groups, but there is currently insufficient data to support such a claim. From our experience, many adolescents report feeling worse after stopping anastrozole in terms of ability to concentrate and overall energy level. This clinical observation may explain that high circulation levels of estradiol affect CNS activity. Further research, however, is needed to make an evidence-based recommendation about optimal indications and duration of anti-estrogen therapy.

TABLE I. Recommended Testosterone Preparations for KS Patients

Testosterone formulation	Dosage	Pharmacokinetics	Monitoring	Side effects
Topical gel	Pre-pubertal: 1 pump (1.25 g gel/day)	High DHT:T ratio	Monitor T level 2 hr after application (after 2 weeks of treatment)	Skin irritation (rare)
Androgel 1% (12.5 mg T/1 pump) (metered-dose pump)	Adolescent: 2 pumps (2.5 g gel/day)			Risk of skin-to-skin transfer
	Once taking ≥3 pumps/day, consider switch to more concentrated form of T gel			
Axiron 2% (30 mg T/1.5 ml) (metered-dose pump with applicator)	30 mg daily (1 pump to axilla)	High DHT:T ratio	Monitor T level 2 hr after application (after two weeks of treatment)	Skin irritation (rare)
	Progressive increase in dosing according to T levels and progression of puberty, assessed every 6 months			Risk of skin-to-skin transfer

As hyperestrogenism in KS is well-accepted, we often recommend a diet low in naturally occurring phytoestrogens.

Partial Androgen Resistance and Compliance

Patients with KS manifest partial androgen insensitivity or resistance, which likely results from increased adipose tissue, aromatization from upregulation of aromatase CYP19 (with enhanced testosterone to estradiol conversion), and decreased activated androgen receptor trafficking from cytoplasm to nucleus. This resistance blunts the physiologic response of KS patients to TRT, and may lead to discouraged patients due to lack of rapid changes in physical appearance. For this reason, it is imperative that the urologist treating patients with KS set age-specific goals for patients regarding muscle strength, facial/sexual hair changes, and penile size changes. Attention to the concerns of the patient with KS is integral to their compliance with the treatment regimen and to ultimate outcome. Compliance must also be assessed by pill counting and weighing of the testosterone container weekly by parents to ensure that the adolescent is properly using the medication. Adolescents with KS, similar to any other group of young men with chronic conditions, have poor recognition of the negative consequences of medical noncompliance. Therefore, close interaction with parents, respect for adolescent autonomy, and regular serial follow-ups with serum blood measurements (T, SHBG, E, LH, FSH, CBC) are critical to achieve treatment objectives. During adolescence, more frequent patient visits may be necessary to maintain and assess compliance, to discuss safety, preventive measures, alcohol/drug use, bullying, depression, and anxiety. An alternative TRT regimen using implantable testosterone pellets for noncompliant patients has been utilized in rare occasions by our group and others [Khera et al., 2009].

Outcomes With TRT and Aromatase Inhibitors

TRT is accompanied by normalization of body proportions, decreased obesity, blood pressure, and improved behavior, work/academic performance, and musculoskeletal development. Additional long-term reduction in autoimmune disease, breast carcinoma, and osteoporosis risk occur [Mehta et al., 1993; Landin-Wilhelmsen et al., 1999]. The long-term consequences of TRT in KS patients and the extent of suppressive effects on the hypothalamic pituitary axis are difficult to assess from available literature. The first report of hormonal therapy outcomes in a large cohort of adolescents with KS was conducted by chart review from our center including 110 adolescent patients with KS treated between 2007 and 2012 (Mehta A, Paduch DA, unpublished work). Patients received hormone therapy (topical TRT (n 110 patients) or aromatase inhibitors (n 75 patients)) initiated at mean age of 13 years. Good clinical efficacy was achieved in all patients as defined by age-specific serum T levels. Following therapy, the percentage of obese patients decreased from 17% to 11% and mean serum T level improved from 240 to 650 ng/dl. Serum LH and FSH increased with puberty progression (2.6 16.6 mIU/ml, and 7 42 mIU/mL, respectively). No adverse outcomes related to TRT were reported. Topical TRT appears safe and efficacious in adolescents with 47, XXY (KS) and was not associated with suppression of serum LH or FSH.

It is unknown whether treatment with testosterone combined with an exercise program and dietary modifications will further lower the risk of developing childhood and adult obesity. Bone mineral density in patients with KS has been shown to correlate positively with serum testosterone, with benefits from testosterone treatment prior to age 20 but not after this age [Kubler et al., 1992; Wong et al., 1993]. The benefits of bisphosphonate therapy have also been demonstrated in decreasing markers of bone turnover in patients with KS [Stepan et al., 2003].

FUTURE DEVELOPMENTS

Gradual and consistent evidence accumulates indicating that early diagnosis and multidisciplinary management of children and adolescents with KS utilizing hormonal supplementation and manipulation to mimic the normal progression of puberty is not harmful and likely beneficial. Three major challenges exist in the endocrinological management of KS: overcoming partial androgen resistance, developing oral androgen receptor agonists, and enhancing understanding of ideal estrogen levels to facilitate optimal physical and neurobiological development. With improved understanding of the molecular defects leading to hypogonadism, and the tissue-specific requirements of sex steroids and growth factors, we will be able to plan and execute randomized prospective multicenter clinical trials which will lead our management of men with KS in the future.

CONCLUSION

KS is the most frequent chromosomal abnormality in infertile males. Physicians should be aware of the variety of issues associated with KS including testicular changes with seminiferous tubules degenerating during pubertal maturation leading to hypogonadism. Early fertility discussion with detection and appropriate management of low testosterone associated with KS has significant potential to avoid the perils of hypogonadism and to have somatic and behavioral benefits along with improvement of general health, pubertal progression, academic progress, and social integration.

ACKNOWLEDGMENTS

This study has been supported by The Frederick J. and Theresa Dow Wallace Fund of the New York Community Trust and Robert Dow Foundation.

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ORIGINAL ARTICLE

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Assessment of adolescent varicocele

Accepted: 9 September 1996

Abstract Varicocele is the most important male factor responsible for decreased fertility potential in married couples. From March through June 1994, 2,470 school boys aged 10–20 years were examined to establish the incidence of consecutive grades of varicocele and to develop a protocol for diagnosis and treatment of adolescents with varicocele. Grade 1 varicocele was found in 18%, grade 2 in 12%, and grade 3 in 5% of the population examined. An original protocol of ultrasonographic (US) examination (previously verified by angioscintigraphy) was introduced to assess boys with clinically diagnosed varicocele. The volume of each testis, testicular volume decrease (TVD), pampiniform vein diameter (PVD), and basal (BBF) and maximum blood flow (MBF) velocities were measured in 625 boys. In 74 cases a semen analysis was performed. The statistical analysis revealed that the presence of venous reflux and PVD correlated with the grade of varicocele. Decreases in testicular volume were highly dependent on the grade of varicocele, PVD, and BBF and MBF velocities. Analysis of the relationship between spermatic (boys over 17 years) and US findings revealed that the quality of spermatogenesis can be predicted by US examination in adolescents with varicocele. The authors recommend multiparametric US examination as a reliable, objective, and repeatable technique for establishing criteria for operative treatment in boys under 18 years of age with varicocele as well as for postoperative evaluation.

Key words Adolescent varicocele · ultrasonographic assessment · Testicular volume decrease · Pampiniform vein diameter · Venous reflux · Semen analysis

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Introduction

Varicocele is among the most common treatable causes of male-related impaired fertility potential. Varicocele is found in 10% to 20% of all males, but in 30%–40% of men who present to infertility clinics [1–3], and can contribute to secondary infertility [4]. Pregnancy rates after varicocelectomy are still low (30%–40%), and not much higher than in untreated males with varicocele [5], probably because this asymptomatic disorder, instead of being diagnosed and treated at the age of 12–15 years when it appears, is usually found a decade later in adult men already having problems with procreation [1, 3, 6–9]. Varicoceles become clinically manifested in 10-year-old boys and increase in incidence with progressive sexual development, reaching a peak incidence of 15% (incidence of the adult male population) by age 14–15 years [1, 10, 11]. Some authors suggest that active case-finding programs can promote early detection and correction of varicocele in adolescents [7, 12].

Taking into account the importance of the problem and inspired by the 1992 World Health Organization (WHO) report [3], we undertook a pilot study of adolescent varicoceles in Polish schoolboys in order to determine the incidence and natural history in adolescents as well as reliable criteria for establishing indications for operative treatment.

Materials and methods

From March through June 1994, 2,470 schoolboys aged 10–20 years enrolled in a randomly chosen cluster of schools in the Pabianice School District (a suburb of Lodz) were examined personally by the authors (J.N. and D.P.) at their schools. Examinations were done with the boy in the upright position using clinical criteria adapted from the classification of Dubin and Amelar [13] in which grade 1 indicates a varicocele detected only by Valsalva maneuver, grade 2 an easily palpable varicocele, and grade 3 a visible varicocele. At the initial evaluation the personal data, varicocele stage, and other abnormalities found were registered on a standardized flow chart. All boys with detected varicoceles were

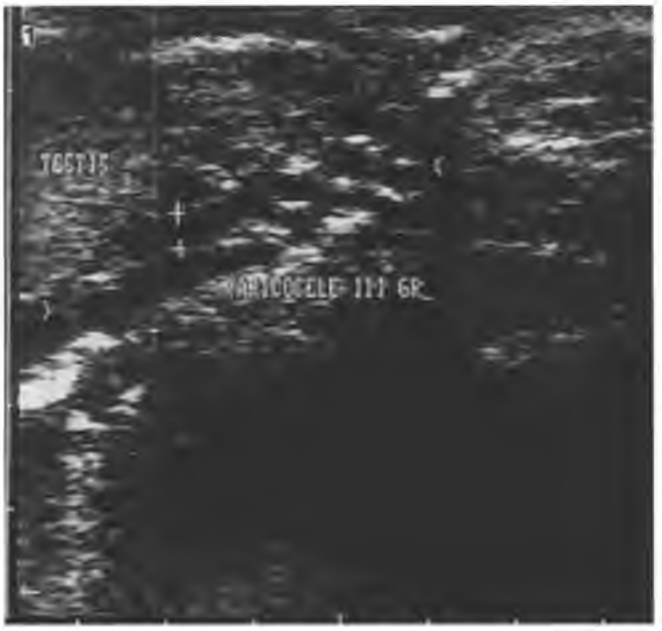

Fig. 1 US scan of testis showing pampiniform plexus with dilated veins above and behind testis (varicocele) with marked diameter of dominant vein

Fig. 2 CW Doppler recording in patient with varicocele showing venous reflux during Valsalva with marked basal (*BBF*) and maximum blood flow (*MBF*) velocities

referred to a university-based adolescent andrology outpatient clinic for further investigation.

In 625 patients ultrasound (US) examinations were performed using the following protocol: (1) three dimensions of each testis were measured with the patient lying supine using a 7.5 MHz linear probe; the testicular volume (TV) was estimated using the formula: TV(ml) = (0.71 × width × length × height)/1,000 and the testicular volume decrease (TVD) of the affected testis was calculated as: TVD(%) = (contralateral testis volume − affected testis volume)/contralateral testis volume; (2) pampiniform vein diameter (PVD) was measured on both sides with the patient standing for at least 2 min; no Valsalva maneuver was employed to increase the veins' diameter and only the diameter of the dominant vein was noted (Fig. 1); and (3) a 3.5 MHz CW Doppler transducer was used to detect venous reflux and determine the velocities of basal blood flow (BBF) and maximum blood flow (MBF) during a Valsalva maneuver (Fig. 2).

In 30 patients nuclear angioscintigraphy was performed to verify the reliability of the authors, newly employed CW Doppler examination technique [14]. For semen analysis, two groups of boys were randomly drawn from the study population: (1) varicocele group (VAR): 36 adolescents, 17–19 years old, with advanced varicocele (grade 2/3 and 3) and otherwise healthy; and (2) control group (NORM): 38 healthy adolescents, 17–19 years old, with no evidence of varicocele. All boys were sexually mature (Tanner stage V) and had no history of genitourinary disease. Two semen samples 2–4 weeks apart were obtained from each patient by masturbation on the seminology lab premises. The required abstinence period was 72 h. Each semen analysis was performed by the same technician manually following the protocol described in the 1992 WHO Laboratory Manual; no CASA equipment was used. Student's *t*-test for differences in means between the two groups, one-way ANOVA, linear regression analysis, correlation tables, and chi-square tests were used for statistical analysis where appropriate. The minimum statistical significance level was alpha = 0.05.

Results

The distribution of the various grades of varicocele detected by clinical examination alone in consecutive age groups is shown in Fig. 3. The overall incidence was: grade 1: 18% (453/2,470), grade 2: 12% (295/2,470), and grade 3: 5% (119/2,470). The incidence of grade 3 reached a plateau at age 13 years, grade 2 at 12, and grade 1 at 10. There were no statistically significant differences in distribution of grades in consecutive age groups; 625 (72%) of 867 referred boys returned for further evaluation. More boys with grade 3 (82%; 98/119) than with grades 1 (71%; 321/453) and 2 (70%; 206/295) came to the outpatient clinic.

The volume of the affected testis (13.33 ml, $n = 625$) was lower than that of the contralateral testis (14.51 ml, $n = 625$), and the difference increased with increasing grade of varicocele (Table 1): TVD was 4% in grade 1,

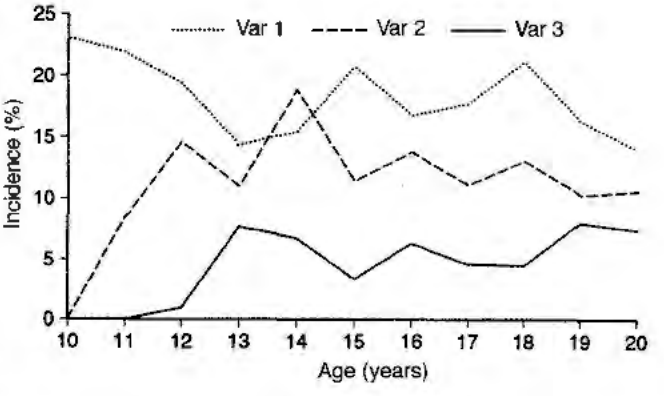

Fig. 3 Incidence of grades of varicocele detected by clinical examination in consecutive age groups

17% in grade 2, and 20% in grade 3 cases. The factorial analysis of variance revealed that the TVD was highly dependent on grade of varicocele, PVD, and the presence of venous reflux, but not on patient age. TVD was greater when both reflux and pampiniform veins were present together (main effect).

Based on results obtained in the control group of 50 boys with no scrotal pathology as well as the PVD of the contralateral testis, a PVD of 2 mm was arbitrarily set as the upper limit of normal (Table 2). Only 16 boys with clinical grade 1 (5%), in contrast to 62 with grade 2 (30%), and 94 with grade 3 varicocele (96%) had a PVD greater than 2 mm. Analysis of regression revealed that PVD depended on the grade of varicocele and the presence of reflux, but not on patient age.

In 51 (16%) of 321 boys with grade 1 varicocele the presence of venous reflux was demonstrated, in contrast to the boys with more pronounced grades: 109 (53%) of 206 boys with grade 2 and 81 (83%) of 98 boys with grade 3 exhibited venous reflux on CW Doppler examination. Mean BBF and MBF velocities are shown in Table 3. The presence of venous reflux correlated only with clinical grade and not with patient age. It can be presumed that in about 10% of healthy adolescents between 10 and 20 years of age the presence of venous reflux can be detected by means of CW Doppler examination. In at least 6% of adolescents presenting with varicocele, PVD as measured by US exceeds 2 mm.

The analysis of semen parameters revealed no statistically significant differences in mean age, semen pH, volume, density, and frequency of spontaneous agglutination between patients from groups VAR and NORM. Statistically significant differences in mean $a+b$-type motility (VAR = 42.4%, NORM = 60.4%), rapid progressive motility (VAR = 15.0%, NORM = 25.0%), numbers of immotile sperm (VAR = 44.5%, NORM = 26.0%), vitality (VAR = 53.6%, NORM = 71.9%), frequency of abnormal sperm morphology (VAR = 15/38, NORM = 23/36), and numbers of sperm with tapered heads (VAR = 16.4%, NORM = 12.0%) were found between patients from groups VAR and NORM. There were statistically significant differences in TV, TVD, PVD, and BBF and MBF velocities between patients from both groups that reflected the pattern found in all boys based on US examination only. Coefficients of correlation were used to determine the degree of association between semen parameters and US findings. There was a clinically and statistically significant linear, negative relationship between sperm motility and both BBF and MBF velocity and PVD. Linear regression analysis revealed that sperm concentration depended on total TV (alpha = 0.05). Although the relationships between other sperm parameters and US measurements were obvious on scatter diagrams, the linear regression significance level exceeded alpha = 0.05, which could be explained by the relatively small group size and the large extent of individual variability in semen parameters.

Table 1 Volume of testes and testicular volume decrease (TVD) in consecutive grades of varicocele (n = 625)

Varicocele grade	Number of patients	Contralateral testis volume (ml)	Affected testis volume (ml)	TVD (%)
1	321	15.5 (SD = 5.4)	14.7 (SD = 5.2)	4 (SD = 15)
2	206	13.4 (SD = 5.6)	11.2 (SD = 5.3)	17 (SD = 16)
3	98	14.2 (SD = 6.6)	11.4 (SD = 5.6)	20 (SD = 13)

Table 2 Pampiniform vein diameter (PVD) in patients with consecutive grades of varicocele (n = 625) and control group (n = 50)

Varicocele grade	Number of patients	PVD contralateral testis (mm)	PVD affected testis (mm)
1	321	1.5 (SD = 0.3)	1.9 (SD = 1.9)
2	206	1.6 (SD = 0.5)	2.2 (SD = 0.5)
3	98	1.7 (SD = 0.3)	3.1 (SD = 0.68)
Controls	50	1.6 (SD = 0.11)	1.7 (SD = 0.14)

Discussion

The management of varicocele in adolescents is still controversial. Although not every case necessarily leads to impaired fertility, due to the prognostic uncertainty affected boys must be considered members of an at-risk group. Some findings of previous varicocele studies are no longer controversial and are generally accepted. Adolescent varicocele is clearly associated with a progressive reduction in TV and abnormal testicular histology (the same changes seen in adults), as well as

Table 3 Velocities of basal (BBF) and maximum blood flow (MBF) in venous reflux to pampiniform veins (n = 625)

Varicocele grade	Number of patients	Contralateral testis velocity (cm/s)		Affected testis velocity (cm/s)	
		BBF	MBF	BBF	MBF
1	321	0.1 (SD = 0.7)	0.1 (SD = 1.3)	1.56 (SD = 3.7)	3.7 (SD = 6.9)
2	206	0.7 (SD = 2.8)	1.24 (SD = 4.2)	6.4 (SD = 7.4)	12.5 (SD = 8.3)
3	98	0.2 (SD = 1.7)	1.7 (SD = 0.3)	10.6 (SD = 5.9)	19.1 (SD = 6.9)

impairment of testicular function [3, 4, 6, 8, 15–17]. Thus, if one accepts that a varicocele may result in testicular growth arrest and male-factor impaired fertility in adults, it would be logical to accept the need for early detection and treatment of varicocele at puberty to prevent progressive and irreversible damage to the testis [3, 7, 8, 9, 12, 16, 17].

Our study was based on mutiparameteric US evaluation of the scrotum to find a simple but reliable, repeatable, and precise diagnostic tool. The results confirm the thesis that varicocele is a common finding in Polish adolescents, with an overall incidence of 35% (including grade 1) using clinical criteria. Though this incidence is higher than the published data of Oster (16%), Steeno et al. (15%), or Pozza et al. (25.8%) [7, 10, 18], we believe that in their studies only moderate and pronounced varicoceles (our grades 2 and 3) were recorded. The total incidence of grades 2 and 3 in our data was 17%, which is similar to the data published by the other authors.

The deleterious effect of varicocele on spermatogenesis is reflected by diminished sperm concentration and motility and increased numbers of sperm with abnormal morphology in spermatograms from infertile males. A similar pattern has been reported in adolescents and young men with varicocele (lower sperm count, motility, and, less frequently, increased numbers of sperm with abnormal morphology). Although spermatograms of adolescents with varicocele often fall within the low normal range, Lipschultz and Goldstein have emphasized that semen parameters in individuals with an unrepaired varicocele can deteriorate over time. Our results of sperm analyses are similar to those published by other authors, although we did not find lower sperm counts in adolescents with varicocele. This could be due to the fact that a varicocele first affects the maturation of spermatozoa and does not seem to interfere with the number of spermatogonia that undergo division in a given period of time. The function of Leydig and Sertoli cells is affected initially causing disturbances in the maturation of germ cells, which are ultimately responsible for the increased numbers of sperm with abnormal morphology.

The clinically significant correlations between semen parameters and US findings demonstrated in our study allow the function of the testes to be predicted based only on US examination, without the necessity of obtaining semen samples. Although further studies are necessary, we believe that our recommended protocol of multiparametric US assessment should be the basic diagnostic tool and should guide therapeutic decisions in adolescent patients with varicocele. Because of the magnitude of the problem, we also suggest that at the age of 15 every boy should be examined by his school or family doctor in order to detect scrotal pathology.

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SEXUAL MEDICINE ORIGINAL RESEARCH

MEN'S SEXUAL HEALTH

Attitudes Toward Penile Transplantation Among Urologists and Health Professionals

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ABSTRACT

Introduction: Penile transplantation, in its infancy, has the potential to reestablish functional outcomes for men with penile loss and disfigurement. However, significant bioethical considerations are pertinent, and systematic discussions are necessary to safely progress implementation.

Aim: To determine the attitude of health practitioners toward the penile transplant and identify the key aspects of concern pertinent to the operation and clinical care.

Methods: Health care professionals from the United States responded to either email invitation, web link, or social media post on Facebook to complete a questionnaire investigating perceptions and attitudes toward penile transplantation.

Main Outcome Measures: Respondents' attitude toward penile transplantation, their own perceived important functions of the penis, and concerns about performing a penile transplantation. Respondents' previous exposure to visceral transplants, to penile disfigurement, and information about penile transplants were used as independent factors in analysis.

Results: Among 412 health care professionals who responded to the questionnaire, 95.9% were in favor of visceral organ transplant, but only 64.3% were in favor of penile transplantation. The results showed that 61.3% of respondents first learned about the penile transplant from mass media, whereas only 37.5% had been exposed through a scientific journal, formal lecture, or a professional colleague. Younger health professionals and those exposed through professional forums surrounding penile transplantation were more likely to be in favor of the procedure ($P < .001$). The most important functions of the penis were identified by respondents as being sexual function (role in sexual activity) and gender identity (being a man) with rates of 86.4% and 85.3%, respectively ($P < .001$). Barriers identified by respondents included the use of immunosuppression and the potential subsequent effect on healthcare resource utilization. Reading an excerpt about penile trauma in war during the questionnaire improved acceptance of penile transplantation (P .05).

Conclusion: Penile transplantation is accepted by most health professionals surveyed. Younger respondents and those informed through professional outlets are more favorable toward penile transplantation. Anticipated limitations include the risk of immunosuppression, lack of available donors, and the effect on healthcare utilization. **Najari B, Flannigan R, Hobgood J, et al. Attitudes Toward Penile Transplantation Among Urologists and Health Professionals. Sex Med 2018;6:316–323.**

Key Words: Penile Transplant; Penile Trauma; Genitourinary Trauma; Penile Reconstruction

Received March 20, 2018. Accepted June 7, 2018.

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https://doi.org/10.1016/j.esxm.2018.06.003

INTRODUCTION

Solid organ transplants have become a foundational therapy for many forms of end-stage organ dysfunction such as hepatic, renal, respiratory, cardiac, and pancreatic failure. These transplants have been shown to decrease mortality, prolong life, and be cost effective in some circumstances, such as renal transplant.[1] Awareness of visceral organ transplantation is increasing as is the frequency; in 2015, a record number of 30,974 solid organ transplants were performed in the United States.[1]

Transplants have been traditionally life-saving, but more recently, the field of organ transplantation has extended to also include organs for the functional benefit. Composite tissue allotransplantation (CTA) is comprised of heterogeneous cadaveric tissues and has advanced the field to include transplantation of organs to restoration, function, structure, and aesthetics.[2] Examples of CTA include face, abdominal wall, larynx, tongue, knees, and penis.[1,3,4] These structures are not life-saving in many cases but potentially contribute to the quality of life of their recipients. Transplant is not without complication, and there are side effects related to the procedure or associated immunosuppression. Thus, assessment of the risk-to-benefit tradeoff for non-life-threatening CTAs is an important consideration and significant controversy exists.[5,6]

Within this realm, the experience and realization of penile transplantation is rapidly evolving. Although guidelines do not yet exist and indications are in its infancy, conceivable indications for men include those with penile amputation or severe disfigurement and functional loss of male genitalia. Here, penile loss may occur most often due to trauma or penile cancer with subsequent penectomy. Because the penis is an external appendage, it is at risk for trauma in military combat. In fact, complex genitourinary injuries have emerged as a common occurrence in current military combat operations.[7] Operation Enduring Freedom (OEF) in Afghanistan and Operation Iraqi Freedom (OIF) have become the longest wars of modern times, resulting in more than 50,000 service members sustaining major injuries. Changes in combat tactics have doubled the rate of genitourinary trauma from 7% to 13% of injured soldiers.[8] Most service members who endure major lower-extremity amputation from IEDs suffer from major genital trauma.[9,10] In fact, between 2001 and 2013, 1,367 U.S. service members sustained genitourinary trauma with 423 (31%) localizing to the penis.[11] Phallic reconstruction using tubularized flaps can be achieved using a microvascular free forearm flap.[12] Sexual function using a variety of penile prosthetics has been reported following reconstruction; however, these are reports limited to populations of sexual reassignment from female to male transsexual and not combat-related injury victims.[12] Sensory perception of the forearm- or tibia-derived penile flap is lost.[12] Appearance of the penile flap is suboptimal, because the flap does not have a distinct glans, although the technique is continually being refined for better cosmetic appearance.

Penile transplantation allows for restoring both urinary and sexual function by providing a highly functional conduit for urination and a "normal"-appearing and functional organ for sexual intimacy. Three reports of human penile transplant have been published in the literature.[13–15] Furthermore, animal studies have been performed conducting penile transplants in beagles with excellent success.[16] There is no question that penile transplantation for men with catastrophic genital loss is both surgically and immunologically feasible and may be a bioethically justified approach to restore quality of life, urinary function, and sexual function. However, penile transplantation is in its infancy and comes with ethical concerns and warranted discussions. We wished to evaluate the perspectives and attitudes of urologists, reconstructive surgeons, and mental health specialists surrounding the use and potential challenges surrounding penile transplantation. Characterizing and comparing these perspectives are important to evaluate present levels of awareness and education among healthcare professionals that may be involved in clinical and surgical care of these patients. Furthermore, results from this study are necessary to form an initial healthcare provider perspective and consensus on pertinent considerations surrounding penile transplantation, and to direct future working groups that are necessary to establish medical, surgical, and ethical guidance to providers involved in penile transplants moving forward.

METHODOLOGY

Institutional review board approval was acquired for conducting this study at Weill Cornell Medicine. Professionals from across the United States, in numerous fields of medicine, predominantly inclusive urology, reconstruction specialists, and mental health specialists, were asked to complete a survey. Inquiries were made via e-mail invitation, web link, or social media post on Facebook. The online survey was sent to members of the American Urological Association (AUA), members of the New York Transplantation Network, and members of the American Society of Reproductive Medicine using the SurveyMonkey platform. Respondents were asked the following: (1) where they learned about penile transplantation; (2) important functions of the penis; (3) were they in favor of organ donation; (4) were they in favor of transplantation of visceral organs that prolong life (ie, kidney); (5) were they in favor of transplantation of organs that improve quality of life (ie, face); (6) were they in favor of penile transplantation; (7) were they in favor of penile transplantation being covered by a veteran's healthcare plan; (8) concern for issues after transplantation; (9) personal experience with friends or family with penile disfigurement; (10) age; (11) gender; (12) religion; (13) race; (14) ethnicity; (15) service to the military; (16) healthcare profession; (17) in favor of penile transplantation; and (18) withdraw from the study. The responses ranged from "Extremely in favor (1)" to "Not at all in favor (5)" (Appendix 1). Effects of reading an excerpt on penile trauma due to war was assessed during the questionnaire by asking respondents to read an excerpt from the book *Beyond the Battlefield: The War Goes on for the Se*verely Wounded by David Wood, which discusses soldiers' experiences and fears of in-field genital injuries (Appendix 2), prior to responding to question 17: "Are you in favor of penile transplantation"?

IBM SPSS Statistics version 24 was used for data analysis (IBM, New York, NY, USA). Categorical responses were converted to numeric integers to test distribution of answers;

because answers were normally distributed, they were described with mean, median, and interquartile ranges. The Mann-Whitney U tests was used to compare binary variables. Comparisons of parameters with 3 or more variables were done using Kruskal-Wallis H test with a Bonferroni correction for multiple tests. Significance was set at $P < .05$ after Bonferroni correction.

RESULTS

411 subjects completed the survey over the course of 3 months spanning from April 2016 to June 2016 (Table 1). Most respondents were in the field of urology, 97.5%.

Perceived Functional Importance of Penis

Gender identity (being a man) and sexual function (role in sexual activity) with "very important" rates of 85.3% and 86.4% respectively were rated as the most important functions of the penis (Figure 1). Erectile function was also deemed to be a highly important penile function but was rated higher among men than women respondents (P .027). Interestingly, respondents aged 35 to 55 and 55 to 74 were more likely than those aged 18 to 34 years to feel that the function of the penis is for gender identity (P .024, P .007 respectively), which may reflect a shift away from phallocentrism in younger populations.

Favor of Penile Transplants

Most respondents were in "extreme favor" of visceral transplantation (ie, kidney, heart, or liver; 77.3%) and organ transplant in general (60.2%); however, these numbers are reduced to 38% for quality-of-life transplants, and 28.1% for penile transplant (Table 2 and Figure 2). Respondents aged over 75 or those with military experience were less likely to be in favor of general organ transplant than any other age group ($P < .01$, P .003 respectively). Individuals with a personal experience with penile disfigurement were more in favor of visceral organ transplant (P .003) but did not differ with respect to support of other transplant types including penile.

Method of Learning

The results showed that 61.3% of respondents first heard about the penile transplant from mass media while only 37.5% had been exposed through a professional means: scientific journal, formal lecture, or a professional colleague. Respondents who learned about penile transplant through professional means were more in favor of penile transplant (P .023), as well as transplants for quality of life (P .043).

Barriers to Penile as Compared to Visceral Organ Transplant

The most concerning barriers identified by the respondents are the fact that immunosuppression is required, a lack of established sources of cadaveric organ donors, and the potential impact penile transplantation could have on healthcare resource utilization (Figure 3). The least concerning topic related to penile transplant was performing penile transplants for "non-life-threatening conditions." Immunosuppression, healthcare resource utilization, and availability of suitable donor sources were the most concerning potential concerns with penile transplantation. The cost and side effects of immunosuppression were more concerning than all other reasons (P .001); while healthcare utilization was more concerning than transplanting in a non-life-threatening condition ($P < .001$).

Psychological aspects associated with penile transplantation related to intimate relationships were of significant concern for responders. Specifically, not identifying graft as "own" with respect to patient (P .009) or partner (P .005) was more concerning than transplanting for a non-life-threatening condition. Shortage of penile cadaveric donors and partner's acceptance of graft were both important concerns for the responders (P .006; $P < .001$).

Table 1. Baseline characteristics of participants completing the study survey

Demographics	Respondents N (%)
Age	
18–34	50 (12.2)
35–54	188 (45.7)
55–74	139 (33.8)
75+	29 (7.1)
Gender	
Male	346 (84.2)
Female	60 (14.6)
Race/Ethnicity	
White/not Hispanic	315 (76.6)
Black	9 (2.2)
White/Hispanic	20 (4.9)
Asian	46 (11.2)
Other	21 (5.1)
Profession	
Urologist	279 (67.9)
Urologist-Reconstruction	40 (9.7)
Urologist-Andrology	34 (8.3)
Other	58 (14.1)
Military Branch	
Air Force	33 (8.0)
Army	27 (6.6)
Navy	20 (4.9)
Other	6 (1.5)
No Military Experience	325 (79.1)
Religion	
Catholic	101 (24.6)
Protestant	97 (23.6)
Jewish	63 (15.3)
None	99 (24.1)
Other	51 (12.4)

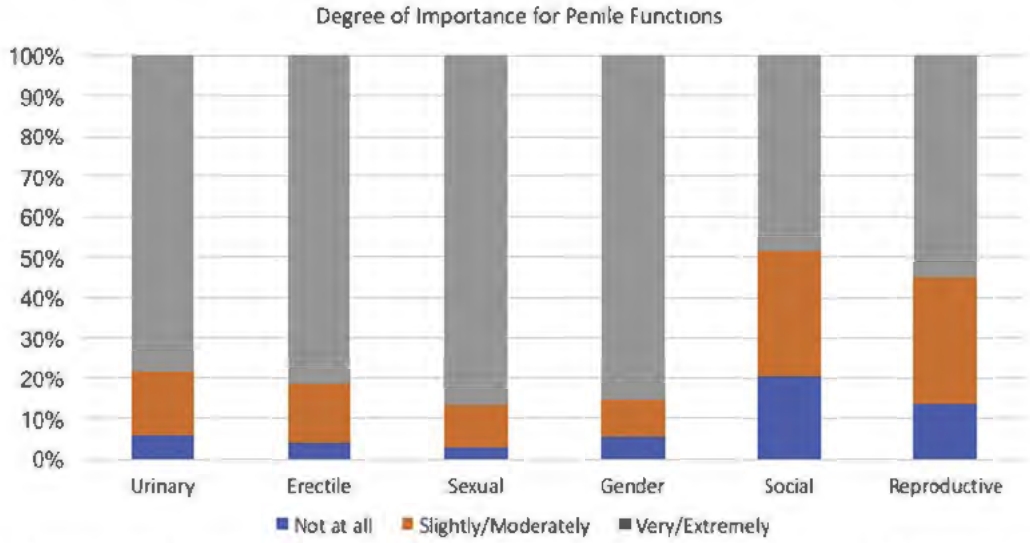

Figure 1. Graph of respondents' answers for their rating of the general importance of each function of the penis (survey question 2). Sexual function followed by gender identity, erectile function, and sexual function were rated most frequently as extremely important functions of the penis. Conversely, social and reproductive function were rated as the least important functions of the penis, significantly less than urinary, erectile, sexual function, and gender identity ($P < .05$).

Coverage of Penile Transplant

The study showed that 65.8% were "very or extremely in favor" of penile transplantation being covered by a veterans healthcare plan. Similarly, respondents that were "not at all in favor" were greatest for penile transplant (8.4%), followed by general transplant (2.5%), quality-of-life transplant (1.2%), and visceral transplant (1.0%) (Figure 4).

Impact of Reading War Excerpt About Penile Trauma

After reading the excerpt from the book *Beyond the Battlefield: The War Goes on for the Severely Wounded* by David Wood (Appendix 2), those in "extreme favor" trended higher from 28.1% to 33.7% (P .16), and those responding "not at all in favor" significantly decreased, 8.4% to 4.8% (P .05; see Figure 4). Furthermore, following the excerpt, respondents that

Table 2. Baseline characteristics of respondents and their favor in penile transplantation

	Respondent in favor of penile transplant? (%)			
	Extremely/Very	Moderately/Mildly	Not at all	*P* value
Total	237 (58.1%)	83 (20.3%)	88 (21.6%)	
Age				.078
18–34	31 (62)	12 (24)	7 (14)	
35–54	100 (53.2)	36 (19.1)	52 (27.7)	
55–74	90 (65.7)	24 (17.5)	23 (16.8)	
75+	14 (48.3)	9 (31)	6 (20.7)	
Gender				.778
Male	202 (58.7)	67 (19.5)	75 (21.8)	
Female	33 (55)	14 (23.3)	13 (21.7)	
Profession				.603
Urologist	156 (56.1)	60 (21.6)	62 (22.3)	
Urologist-reconstruction	21 (53.8)	7 (17.9)	11 (28.2)	
Urologist-andrology	22 (64.7)	5 (14.7)	7 (20.6)	
Other	38 (66.7)	11 (19.3)	8 (14.0)	
Military experience				.325
Yes	44 (52.4)	17 (20.2)	65 (20.1)	
No	193 (59.6)	66 (20.4)	23 (27.4)	
Personal experience with penile disfigurement				.176
Yes	121 (56)	40 (18.5)	55 (25.5)	
No	112 (60.5)	40 (21.6)	33 (17.8)	

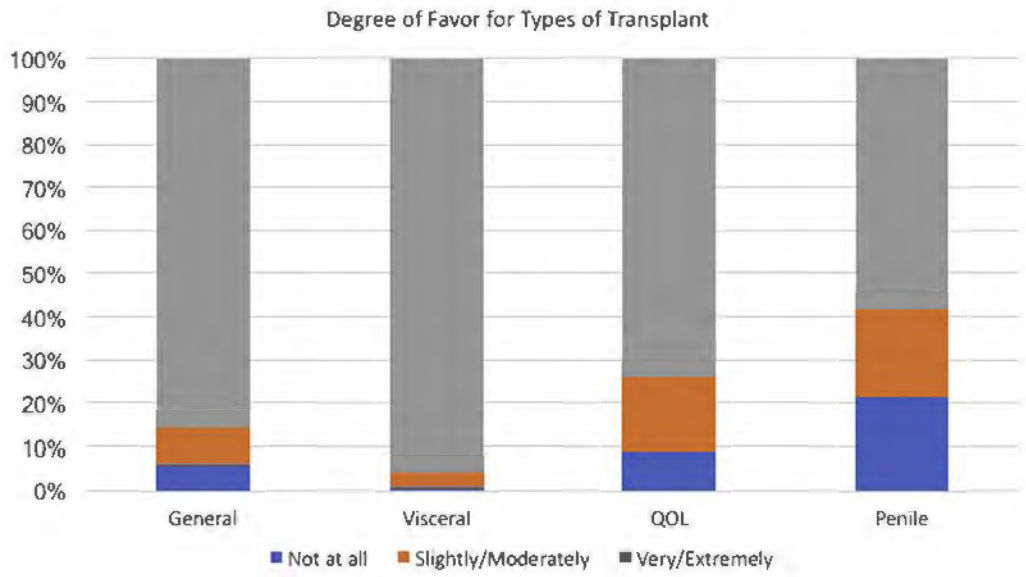

Figure 2. Graph of respondents' favorability toward organ transplant in general, visceral organ transplant, transplants to improve quality of life, and penile transplant. Respondents were most likely to be extremely in favor of visceral organ transplant followed by transplantation in general. Penile transplantation had the least number of responses in extreme favor and was the most likely to have responses of either mildly in favor or not at all in favor, Kruskal-Wallis $P < .001$.

had learned through a professional source were more likely to be in favor of penile transplant than those learning through mass media (P .005).

DISCUSSION

As therapeutic options progress for men with traumatic penile amputation and disfigurement, consideration of both the technical feasibility, as well as the social and ethical implications are vitally important. Technical feasibility hinges on the success of surgical reconstruction and immunosuppression. Surgical reconstruction requires appropriate anastomosis of the urethra, corpus spongiosum, corpus cavernosum, dorsal artery, superficial and deep dorsal veins, dorsal nerve, fascia, and skin.[2] It is unclear if the anastomosis of cavernosal arteries are of benefit.[17] These techniques were initially devised from penile reimplantation,

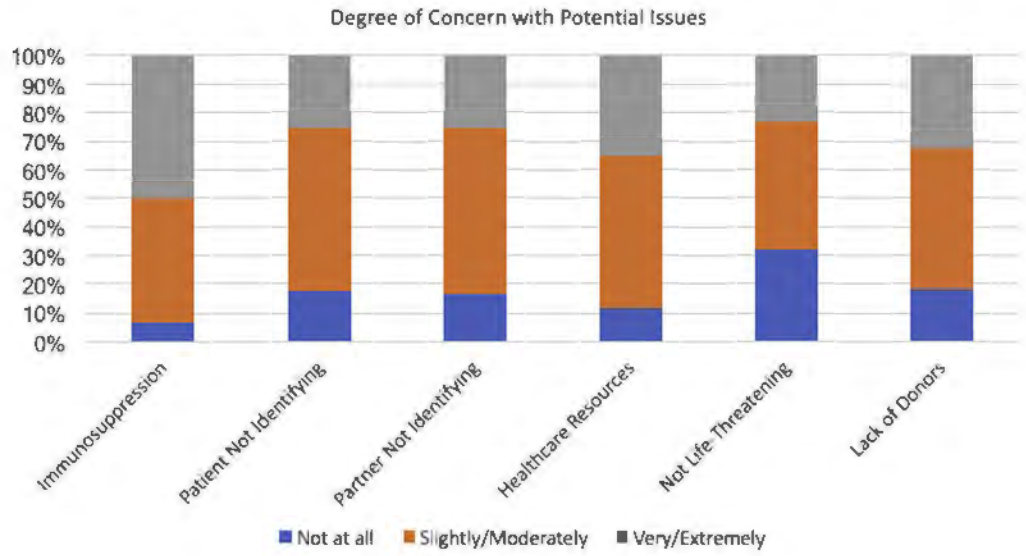

Figure 3. Barriers to transplant. Respondents' concerns expressed for immunosuppression, identifying their graft as not their own, partner not identifying their graft as the patient's, healthcare resource utilization, non-life-threatening condition, or lack of donors. Immunosuppression, healthcare resource utilization, and lack of organ donors were the most concerning potential concerns with penile transplantation. Immunosuppression was more concerning than all other reasons ($P = .001$); while healthcare utilization was more concerning than transplanting in a non-life-threatening condition ($P = .01$) were all more concerning than transplanting for a non-life-threatening condition.

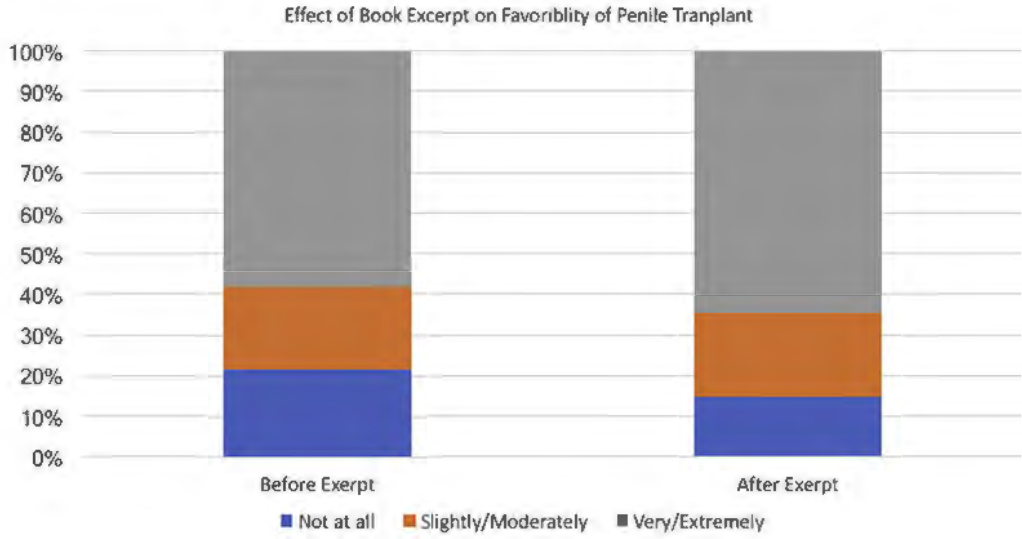

Figure 4. Respondents' responses surrounding their favor of penile transplant at baseline prior to reading the excerpt from the book *Beyond the Battlefield: The War Goes on for the Severely Wounded* by David Wood (Appendix 2) and following reading this excerpt. Pooling responses demonstrate that attitudes were more in favor following having read the excerpt ($P = .025$).

where 28 cases have been reported in the literature, 16 with complications noted. Three penile transplants have been performed to date. Each was technically successful with respect to graft survivability. The first, performed in Guangzhou General Hospital on a 44-year-old man with traumatic penile loss, resulted in a viable graft with some distal penile skin necrosis.[13] However, after 14 days the patient and his wife requested extirpation of the graft because of psychological distress of the aesthetics. No rejection was identified on pathologic examination. The second case, performed in Tygerberg Hospital South Africa for a 21-year-old man with penile amputation secondary to circumcision, required extensive dissection to identify a viable vascular source for the graft using the inferior epigastric and superficial external pudendal arteries. Postoperatively, ancillary procedures were required for thrombosis of the penile artery, infected hematoma, and urethral fistula. The resultant condition is improved psychologic condition, intact penile skin sensation, and erectile function.[14] The third, performed at Massachusetts General Hospital in Boston, MA, on a 64-year-old man who underwent penectomy for penile cancer several years prior. The procedure was 15 hours in duration but resulted in a functional graft with the capability to void but unknown erectile function at this point. An episode of rejection was treated without further complication.[15]

Standard immunosuppression regimens have not been developed specifically for penile transplantation to date. However, 2 published single recipient reports and limited animal data indicate that minimal immunosuppression is needed to prevent the rejection of penile CTA. Based on a single report, standard immunosuppression protocol for renal transplantation (induction with Alemtuzumab followed by tacrolimus (FK-506) and mycophenolate mofetil) seems to be adequate immunosuppression to start in penile CTA transplant. Further studies are needed to assess how the potential benefits of penile transplant compare to the long-term risks of immunosuppression.[18] It is known that the recipient's motivation, compliance, and psychiatric issues affect the outcome of solid organ transplantation.[19] Currently, very limited data exists about the psychological profile of men with total penile and genital loss, as well as the views, attitudes, and beliefs of healthcare providers surrounding penile transplant. This study specifically addresses the latter.

An overwhelming support for visceral organ transplant was noted from the respondents (95.9%). These procedures are often life-saving, involving the heart, lungs, and liver, but they may come at a significant cost to the healthcare system. Other visceral organ transplants such as kidney transplants prolong and improve quality of life and are cost effective compared with the alternative treatment of dialysis.[1] In contrast to visceral organ transplant, respondents were less likely to be in "extreme favor" of CTA transplants for quality-of-life benefit (38.0%) and even less for penile transplant (28.1%), and more were likely to be "not in favor" (8.4%) among penile transplants compared with quality-of-life transplants (1.2%), and visceral transplant (1.0%). This may be due to the presumed risk-to-benefit trade-off perceived by respondents because the primary function of the penis was felt to be sexual function, gender, and erectile and urinary function compared with visceral organs that are required to live. This, coupled with the greatest concerns for penile transplant being immunosuppression and healthcare utilization, it may be felt that penile transplant may lead to health complications that may significantly affect both the recipient's quality and longevity of life in addition to required additional healthcare resources to manage the acquired comorbidities. Furthermore, younger respondents aged 18 to 34 were more likely to be in "extreme favor" of penile transplants (40%) compared with older respondents, which reported similar rates beyond age 35 (age

35–54: 26.2%; age 55–74: 27.9%; age 75+: 20.7%). This may reflect the relative importance of penile function during younger men who may be more focused on sexual relations and reproduction.[20 22] Younger respondents aged 18 to 34 place less importance of the penile function serving as gender identity compared with the older cohorts. This may be a reflection of more awareness of gender diversity among younger cohorts. An alternative explanation may be a shift in phallocentrism ("phallus masculinity") among millennials. Because respondents older than 75 are less likely to be supportive of organ transplant in general, it may be that older individuals are less accepting of transplantation in general based on their beliefs, understanding, or experiences. Another hypothesis for why penile transplants are not as favored as visceral organ transplants is that most respondents are likely not as well informed with penile transplantation as they are with other forms of more time-tested and established visceral transplantation. For instance, 61.3% of respondents first learned about penile transplantation through mass media, but only 37.5% had been exposed through a scientific journal. Supporting this hypothesis, our results demonstrate that respondents who had learned about penile transplantation through a professional outlet were more likely to favor penile transplant and those transplants working to improve quality of life for these individuals.

Our ability to fully appreciate the extent of the negative effect associated with traumatic loss of one's penis may be difficult to appreciate, and therefore it is difficult to weigh the potential harms and benefits of undergoing a penile transplant. Our data support this hypothesis, because respondents were found to be more in favor of penile transplantation after reading the excerpts of soldiers' thoughts and experiences who have had complex genital trauma. However, this is likely related to empathy and appreciation of the internal psychological struggle of these men, because simply having a personal experience with a friend or family suffering from penile disfigurement did not alter respondents' opinion of penile transplantation.

The results of this study are limited to healthcare professionals and predominately urologists. The insight into the favorability, perceived functions of the penis, and potential challenges with penile transplantation is important to shape future discussions and studies. Furthermore, results from this study are necessary to form an initial healthcare provider perspective and consensus on pertinent considerations surrounding penile transplantation, and to direct future working groups that are necessary to establish medical, surgical, and ethical guidance to providers involved in penile transplants moving forward. Future study populations of interest would include andrologists who perform penile surgery, transplant surgeons, and transplant medicine teams who are directly involved with immunosuppression and the associated complications.

This work has purposefully omitted the study and attitudes toward penile transplantation from the perspective of potential family members and loved ones who would consent to donating intimate organ such as the penis. However, such studies are required to determine the willingness of donor families to determine potential number of suitable organs available for transplantation. Furthermore, targeting the potential patient populations; men with traumatic penile injuries and those having undergone penectomy for penile cancer would add further insight. Previous studies have demonstrated that penectomies have a considerable impact on men's sexual function and relationships, urinary function, masculinity, and mental well-being[23]; similarly, among U.S. military service men with genitourinary (GU) trauma, 40.1% report post-traumatic stress disorder compared with 22.6% without GU trauma, 46% vs 27% report chronic pain, 15% vs 6% report sexual dysfunction, 19.3% report major depression vs 7.1%, 19.6% vs 9.3% report substance abuse, 3.3% vs 1.0% report panic disorder, and 77.5% vs 1.9% have seriously contemplated suicide. It is clear that penile loss in both groups of men results in significant psychological distress and severe impairments to quality of life. However, it will be important to evaluate how these men balance the quality-of-life impairments compared with their perceptions of the potential risks and complications associated with penile transplantation.

CONCLUSION

With the advancement of the penile transplant programs, systematic protocols, and appropriate patient selection criteria on an individual basis, it is theoretically possible to significantly improve function and quality of life in select men. Our study demonstrates that the majority of health professionals are in favor of penile transplantation, albeit less than visceral organ transplantation. The most concerning potential barriers to penile transplantation include the requirement of immunosuppression, potential shortage of donors, and impact on healthcare utilization. Further research is required to assess these concerns, as well as the views of men with a history of penile amputation or disfigurement before widespread implementation.

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Conflict of Interest: The authors report no conflicts of interest.

Funding: This study was funded by the Robert Dow Foundation.

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Category 3

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://doi.org/10.1016/j.esxm.2018.06.003.

BJUI BJU INTERNATIONAL

Factors associated with ejaculatory and orgasmic dysfunction in men with erectile dysfunction: analysis of clinical trials involving the phosphodiesterase type 5 inhibitor tadalafil

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Accepted for publication 21 April 2011

Previous presentation: Selected data from these studies were presented at the 13th Congress of the European Society for Sexual Medicine, November 14–17, 2010, Malaga, Spain

Study Type – Prognosis (cohort series)
Level of Evidence 2a

What's known on the subject? and What does the study add?
Many men with ejaculatory dysfunction (EjD) or orgasmic dysfunction (OD) do not seek medical attention. In fact, relatively high proportions of men with erectile dysfunction (ED) have abnormal EjD or OD, and even mild ED is associated with them. Because of the overlap of ED, EjD, and OD, the presence of any one condition should prompt questions about, or a work-up for, the other two.

OBJECTIVE

- To determine frequencies of, and risk factors for, ejaculatory dysfunction (EjD) and orgasmic dysfunction (OD) in men with different degrees of erectile dysfunction (ED).

PATIENTS AND METHODS

- Baseline data from 28 ED trials were integrated and analysed.
- The International Index of Erectile Function Question 9 (IIEF-Q9; 'When you had sexual stimulation or intercourse, how often did you ejaculate?') and IIEF-Q10 ('How often did you have the feeling of orgasm with or without ejaculation?') were used to evaluate ejaculatory and orgasmic functions.
- Responses of 'almost never or never' or 'a few times (much less than half the time)' were taken as evidence of EjD or OD, respectively, whereas responses of 'almost always or always' or 'most times (much more than half the time)' were taken as evidence of normal function.
- Estimates of the relative risks (RRs) of EjD or OD were determined for multiple patient characteristics.

RESULTS

- Among 12 130 study participants with available data, only 5117 (42.2%) reported normal ejaculatory function, and 4321 (35.6%) normal orgasm, regardless of ED severity.
- Among subjects with poor ejaculatory function, 16.7% had mild ED, and among subjects with poor sensation of orgasm, 21.9% had mild ED.
- Frequencies of EjD and OD increased with increasing ED severity. Of the 5117 individuals with normal ejaculatory function, 796 (15.6%) had poor sensation of orgasm. Of the 4321 subjects with normal orgasm, 226 (5.2%) had poor ejaculatory function.
- Men with (vs without) EjD or OD tended to be younger: 53.7 vs 56.9 years and 54.2 vs 56.2 years, respectively.
- Factors associated with increased RRs of EjD and OD included cardiomyopathy (RR for EjD 1.74; RR for OD 1.59); cardiac failure (RR 1.40; 1.22); and baseline use (or history of use) of antipsychotics (RR 1.45; 1.30), selective serotonin reuptake inhibitors (RR 1.31; 1.27), and tricyclic antidepressants (RR 1.34; 1.28).

CONCLUSIONS

- EjD and OD occurred at baseline in more than one in three men enrolled in tadalafil trials. Even men with mild ED reported EjD or OD.
- Further studies are warranted to better understand the impacts of EjD and OD on male sexuality and quality of life.

KEYWORDS

epidemiology, erectile dysfunction, human, male, orgasm, phosphodiesterases

INTRODUCTION

Oral phosphodiesterase type 5 (PDE5) inhibitors are effective, well-tolerated, first-line treatments for erectile dysfunction (ED) [1–3]. Pivotal clinical trials of PDE5 inhibitors evaluated ED treatment efficacy chiefly using the erectile-function (EF) domain of the International Index of Erectile Function (IIEF) [4–7]. However, in recent years attention has shifted toward a broader spectrum of patient- (and partner-) centered outcomes, including sexual satisfaction, psychosocial variables (e.g. confidence, self-esteem, relationship quality) and other factors potentially related to quality of life [8–14]. In particular, both orgasmic function (OF) and ejaculatory function (EjF) have occupied an increasingly salient place in recent research on male sexual dysfunction. For example, studies in heterosexual couples showed that lasting sexual satisfaction was closely linked to the likelihood of orgasm, which was in turn also related to emotional intimacy and relationship satisfaction [15,16].

Ejaculatory dysfunction (EjD) includes premature ejaculation (PE), which is a frequent cause of distress and relationship discord [17,18]. Probably less frequent, but not unimportant, are delayed or retarded ejaculation (increased time to ejaculation), anejaculation (inability to ejaculate), painful ejaculation, retrograde ejaculation, as well as a reduced volume of ejaculate or diminished force of ejaculation [19]. Like ED, EjD is among the most common forms of male sexual dysfunction: by one estimate, 25–40% of men experience EjD at some time in their lives [20]. However, EjD is also an infrequent precipitant of medical or sexological consultations [21–23]. EjD is also often comorbid with ED and/or reduced sexual desire [24,25]. Noting that EjD may develop secondary to ED, consensus treatment panels have recommended that ED and other forms of sexual dysfunction should be treated first, contending that EjD may be mitigated via effective management of concomitant ED [1,26].

Consensus guidelines, systematic reviews, and randomized controlled trials (RCTs) have suggested that there are potential benefits of PDE5 inhibitors, either alone or in tandem with other therapies (e.g. selective serotonin reuptake inhibitors [SSRIs]), in managing EjD [26–29]. However, most studies have concentrated on PE and not on delayed ejaculation, decreased volume of ejaculate, or reduced force of ejaculation. Because most clinical publications have focused on PE, and comparatively less is known about other forms of EjD, aims of our recent research have included achieving a better understanding of the prevalence of EjD and orgasmic dysfunction (OD), as well as of potential demographic and clinical risk factors for these conditions and their pathophysiological mechanisms. Objectives of the present exploratory *post hoc* analysis of baseline characteristics in tadalafil clinical trials were to determine the prevalence of EjD and OD in men with variable degrees of ED (and characterize the degree of overlap between and among these conditions); evaluate relationships between EjD and OD across distinct ED severities; and identify potential demographic and clinical risk factors for these conditions.

PATIENTS AND METHODS

STUDY DESIGN

This pooled-data analysis included 28 RCTs involving ED treatment with tadalafil (5–20 mg as needed or 2.5–5.0 mg once daily) or placebo for ≥12 weeks, including 26 studies of men with ED in the general population and two studies of men with diabetes mellitus (Internal Study Identifiers H6D-MC-LVBK [30] and H6D-MC-LVFZ, ClinicalTrials.gov identifier NCT00547183; once-daily dosing [31]). In five studies, men with ED received once-daily tadalafil: Internal Study Identifiers H6D-MC-LVFP, ClinicalTrials.gov identifier NCT00381732; H6D-MC-LVCV (evaluated 10-mg dose); H6D-MC-LVFZ; H6D-MC-LVGH, ClinicalTrials.gov identifier NCT00422734; and H6D-MC-LVHX, ClinicalTrials.gov identifier NCT00836693. The present analysis included study sites in North and South America, Europe (including Russia), the Middle East, Asia, and Australia. Because this analysis involved baseline characteristics exclusively, and no post-baseline data were included, data from the foregoing studies were pooled, even though certain studies differed in design and included both placebo-controlled and open-label (e.g. crossover) trials.

ELIGIBILITY CRITERIA AND STUDY POPULATIONS

Men aged ≥18 years with mild, moderate, or severe ED of psychogenic, organic, or mixed causes were eligible for inclusion in the base studies, provided that they had ED of ≥3 months' duration within a heterosexual relationship. Excluded from these studies were any men who had unstable angina pectoris or were using nitrates.

OPERATIONAL DEFINITIONS OF EJD AND OD

At baseline, study participants were asked to answer questions on the IIIEF [7] for the previous 4-week, treatment-free, run-in period. Response options included (0) 'no sexual stimulation or intercourse', (1) 'almost never or never', (2) 'a few times (much less than half the time)', (3) 'sometimes (about half the time)', (4) 'most times (much more than half the time)', and (5) 'almost always or always'. Based on responses to IIEF question 9 (IIEF-Q9) and IIEF-Q10, we operationally defined EjD and OD, as well as normal EjF and OF.

For EjD, men were asked IIEF-Q9: 'Over the past 4 weeks, when you had sexual stimulation or intercourse, how often did you ejaculate'? Responses of 'almost never or never' or 'a few times (much less than half)' were taken to indicate EjD, whereas responses of 'almost always or always' or 'most times (much more than half the time)' signified normal EjF. For OD, men were asked IIEF-Q10: 'Over the past 4 weeks, when you had sexual stimulation or intercourse, how often did you have the feeling of orgasm with or without ejaculation'? Responses of 'almost never or never' or 'a few times (much less than half)' were taken to indicate OD, whereas responses of 'almost always or always' or 'most times (much more than half the time)' signified normal OF.

STATISTICAL METHODS

Numbers and proportions of men with different responses of EjF and OF, and other descriptive statistics, were computed. To evaluate risk factors for EjD or OD, we constructed 2 × 2 contingency tables for baseline sociodemographic characteristics, clinical comorbidities/disease profiles, and medication use (or history of use). The relative risk (RR) of having EjD or OD in a subgroup (relative to those not in the

subgroup) was estimated. The 95% CIs for RRs were from methods suggested by Thomas [32]; associated two-sided *P* values were from chi-squared tests. To avoid over-interpretation of analysis based on such a large sample of subjects, only those risk factors with a RR of ≥1.20 or <0.80 with an associated chi-square $P < 0.05$ were considered significant. All analyses performed to support this disclosure were *post hoc* and exploratory in nature.

RESULTS

DEMOGRAPHIC CORRELATES

Men with (vs without) EjD or OD tended to be younger than their counterparts without these conditions: mean ages of 53.7 vs 56.9 years for EjD and 54.2 vs 56.2 years for OD (both $P < 0.001$). Among the 12 130 study participants with both IIEF-Q9 and IIEF-Q10 data, individuals of Asian descent had significantly higher frequencies (both $P \leq 0.001$) of self-reported EjD or OD compared with their counterparts of other racial origins (Table 1). In particular, Asians were ≈34% more likely to have EjD and 33% more likely to have OD (vs their non-Asian counterparts) (Table 1).

Study participants residing in Asia, including the Pacific Islands and Japan, were also significantly more likely to have EjD or OD than those residing in other regions: by 40% to 54% for EjD and 37% to 43% for OD ($P < 0.001$ for each; Table 1).

TABLE 1 Demographic characteristics associated with EjD and OD

Demographic characteristic	*N*	Relative risk (95% CI)
Associated with EjD:	10 167	
Race:		
Asian	570	1.34 (1.26–1.43)
Geographic region:		
Asian/Pacific island site	479	1.40 (1.31–1.49)
Japanese site	308	1.54 (1.44–1.64)
Associated with OD:	10 091	
Race:		
Asian	557	1.33 (1.26–1.40)
Geographic region:		
Asian/Pacific island site	469	1.37 (1.30–1.44)
Japanese site	307	1.43 (1.35–1.51)

TABLE 2 Distribution of EjF severities across different degrees of OF severities

	EjF severity, *n* (%)				
OF	No intercourse	Poor	Half the time	Good	Total
No intercourse	222 (79.3)*	27 (0.5)	2 (0.1)	11 (0.2)	262
Poor	40 (14.3)	4456 (88.3)	469 (27.8)	796 (15.6)	5 716
Half the time	10 (3.6)	336 (6.7)	996 (59.0)	444 (8.7)	1 786
Good	8 (2.9)	226 (4.5)	221 (13.1)	3866 (75.6)	4 321
Total	**280**	**5045**	**1688**	**5117**	**12 130**†

**Percentages in parentheses are based on column totals and do not necessarily sum to 100 due to rounding. †N = 12 130 participants in tadalafil ED trials who had a response for EjD and OD.*

TABLE 3 Distribution of OF severities across different degrees of EjF severities

	OF severity, *n* (%)				
EjF	No intercourse	Poor	Half the time	Good	Total
No intercourse	222 (84.7)*	40 (0.7)	10 (0.6)	8 (0.2)	280
Poor	27 (10.3)	4456 (77.4)	336 (18.8)	226 (5.2)	5 045
Half the time	2 (0.8)	469 (8.1)	996 (55.8)	221 (5.1)	1 688
Good	11 (4.2)	796 (13.8)	444 (24.9)	3866 (89.5)	5 117
Total	**262**	**5761**	**1786**	**4321**	**12 130**†

**Percentages in parentheses are based on column totals and do not necessarily sum to 100 due to rounding. †N = 12 130 participants in tadalafil ED trials who had a response for EjD and OD.*

DISTRIBUTIONS OF EJD, OD AND ED (DEGREES OF OVERLAP)

Among 12 130 study participants in tadalafil ED clinical trials who had an EjD and an OD response, 5117 (42.2%) reported normal ejaculatory responses, and 4321 (35.6%) normal orgasmic responses (Tables 2,3). Of the 5117 study participants with normal EjF, 796 (15.6%) had poor orgasmic responses (OD; Table 2). Conversely, of the 4321 subjects with normal OF, 226 (5.2%) had poor ejaculatory responses (EjD; Table 3). Thus, the presence of normal ejaculation did not preclude diminished sensation of orgasm in men.

In all, 4805 (39.6%) study participants with measures of baseline ED severity and a response for EjD were diagnosed with mild, 3175 (26.2%) with moderate, and 4138 (34.1%) with severe ED. The frequencies of poor ejaculatory and orgasmic responses were highly correlated with ED severity. Among subjects with EjD, 16.7% had mild ED, 30.1% had moderate ED, and 53.2% had severe ED (Table 4). Among subjects with OD, 21.9% had mild ED, 29.5% had moderate ED, and 48.6% had severe ED (Table 5).

BASELINE COMORBID CONDITIONS AND CONCOMITANT (OR PRIOR) MEDICATIONS ASSOCIATED WITH EJD OR OD

Comorbid risk factors significantly associated with an increased or decreased likelihood of EjD are presented in Fig. 1A and medication factors in Fig. 1B. Corresponding data for OD are presented in

TABLE 4 Distribution of EF severities across different EjF severities

	EF severity, *n* (%)			
EjF	Mild	Moderate	Severe	Total
No intercourse	6 (2.1)*	23 (8.2)	251 (89.6)	280
Poor	841 (16.7)	1518 (30.1)	2678 (53.2)	5 037
Half the time	814 (48.3)	578 (34.3)	292 (17.3)	1 684
Good	3144 (61.4)	1056 (20.6)	917 (17.9)	5 117
Total	**4805**	**3175**	**4138**	**12 118**†

**Percentages in parentheses are based on column totals and do not necessarily sum to 100 due to rounding. †N = 12 118 participants in tadalafil ED trials who had a response for EjD.*

TABLE 5 Distribution of EF severities across different OF severities

	EF severity, *n* (%)			
OF	Mild	Moderate	Severe	Total
No intercourse	8 (3.1)*	17 (6.5)	236 (90.4)	261
Poor	1262 (21.9)	1698 (29.5)	2796 (48.6)	5 756
Half the time	895 (50.3)	582 (32.7)	304 (17.1)	1 781
Good	2641 (61.1)	877 (20.3)	804 (18.6)	4 322
Total	**4806**	**3174**	**4140**	**12 120**†

**Percentages in parentheses are based on column totals and do not necessarily sum to 100 due to rounding. †N = 12 120 participants in tadalafil ED trials who had a response for OD.*

*TABLE 6 RRs of baseline comorbid factors and medications that were >1.20 but not significantly associated with EjD or OD**

	RR (95% CI)	*P*
Not significantly associated with EjD*:		
Baseline comorbid conditions:		
Other cerebrovascular disease	1.40 (0.97–2.01)	0.157
Renovascular disease	2.02 (1.98–2.06)	0.314
Baseline medications:		
Other hypertension drugs	1.21 (0.80–1.83)	0.422
Serotonin-norepinephrine reuptake inhibitor	1.99 (1.95–2.02)	0.321
Not significantly associated with OD†:		
Baseline comorbid conditions:		
Other cerebrovascular disease	1.46 (1.13–1.88)	0.067
Renovascular disease	1.75 (1.72–1.78)	0.386
Vasculitis	1.31 (1.04–1.66)	0.077
Baseline medications:		
Other hypertension drugs	1.23 (0.82–1.84)	0.411

**N = 10 167 study participants had available data. †N = 10 091 study participants had available data. 'Other cerebrovascular disease' was defined as cerebrovascular disease other than ischaemic or haemorrhagic. 'Other hypertension drugs' were defined as medications other than α-blockers, β-blockers, calcium channel blockers, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, diuretics, and centrally acting sympathomimetics.*

Fig. 1C (baseline comorbid factors) and Fig. 1D (baseline/prior medications). Baseline clinical factors that were not significantly associated with EjD or OD but with RR values that were >1.20 are presented in Table 6.

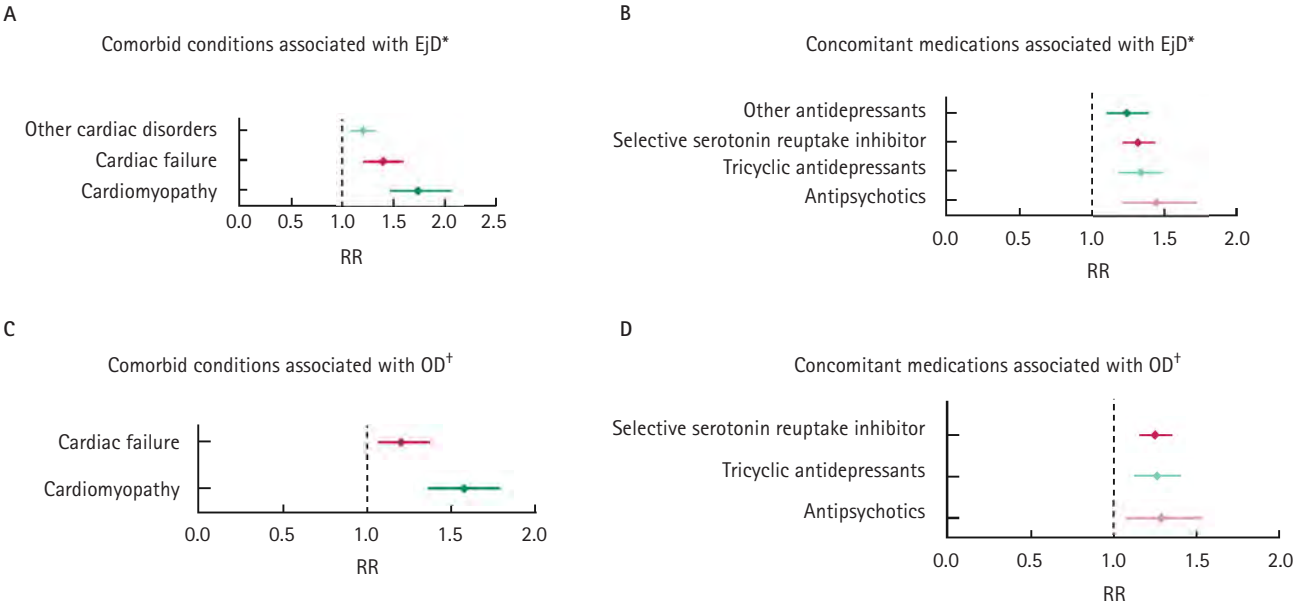
Leading baseline clinical risk factors for EjD and OD included cardiomyopathy (RR for EjD 1.74; RR for OD 1.59) and cardiac failure (1.40; 1.22, respectively) (Fig. 1). Concomitant medications significantly associated with increased RRs of EjD and OD included SSRIs (RR 1.31; 1.27), tricyclic antidepressants (1.34; 1.28), and antipsychotics (1.45; 1.30) (Fig. 1). Men with a history or concomitant use of antidepressants (RR for EjD 1.23) were also significantly more likely to have EjD compared with their counterparts without a history of such treatment.

DISCUSSION

The present pooled-data analysis of baseline characteristics in clinical ED trials involving tadalafil suggested that male sexual dysfunction transcends ED, with normal OF or EjF being potentially as important as the quality of erection in determining men's sexual satisfaction. In particular, about one of every five men who had mild ED reported poor OF or EjF, suggesting that these problems are common in the general population of adult men. Taken together, the substantial frequency of EjD and/or OD in men with ED, and the extensive overlaps among these conditions, underscores the importance of maintaining an increased index of clinical suspicion for the presence of one condition when the other has been diagnosed.

On the other hand, that poor ejaculatory and orgasmic responses were increasingly likely across increasing strata of ED severity suggests that ED remains an important impediment to overall sexual function. In the present analysis, men with a history of cardiovascular disease, particularly cardiomyopathy, were at significantly advanced RR (by 59%–74%) of EjD and OD. Concomitant medications significantly associated with EjD or OD included antidepressants (SSRIs, tricyclic antidepressants) and antipsychotics. Men of Asian descent were significantly more likely to have EjD or OD. Study participants

*FIG. 1. RRs of selected baseline comorbid conditions (**A**) and concomitant medications (**B**) significantly associated with EjD. RRs of selected baseline comorbid conditions (**C**) and concomitant medications (**D**) significantly associated with OD. Data are RRs (filled circles) ± 95% CIs (bars). Factors with RRs ≥ 1.20 or <0.80 with an associated chi-square P < 0.05 were considered significant. *N = 10 167, †N = 10 091 study participants had data available. 'Other antidepressants' were defined as antidepressants in classes of medication other than SSRIs, selective norepinephrine reuptake inhibitors, tricyclic antidepressants, or monoamine oxidase inhibitors. These agents included bupropion, maprotiline, mirtazapine, nefazodone, and trazodone. 'Other cardiac disorders' were defined as those other than ischaemic heart disease, arrhythmias, cardiac failure, and cardiomyopathy.*



residing in Asia, including the Pacific Islands and Japan, were also significantly more likely to have EjD or OD. These findings are consistent with previous epidemiological research concerning sexual satisfaction in these populations [33,34]. Further research is warranted to better characterize the complex interplay of genetic susceptibility, prevalences of predisposing conditions or medications, cultural determinants, or a combination of these and other factors in determining male sexual dysfunction. Unlike the case with ED, men with EjD or OD tended to be younger than their counterparts without these conditions.

Many of the present findings are consistent with previously reported data. For the sociodemographic factors of patient age and race, a recent population study of >3000 younger men (mean [range] age 29.9 [18–48] years) showed that age and the duration of heterosexual relationships were significantly associated with self-reported ejaculation latency time [35]. Other studies have suggested that men with rapid ejaculation tended to be younger (and overall healthier) than those with ED [36]. The EjD literature suggests that PE is also more likely to have an inherited component than delayed ejaculation, which is a largely maladaptive response [35,37,38].

What is new in our present study is the large number of patients analyzed and the focus on lack of ejaculation. Most studies focus on PE; however, we chose IIEF-Q9, which asks only about inability to ejaculate, thus eliminating PE from the analysis.

It is also striking that, contrary to common belief, men did experience OD, and the presence of ejaculation was not equivalent to experiencing orgasm, as up to 15.6% of men with normal EjF reported no or decreased sensation of orgasm. This finding underscores the importance of differentiation between orgasm and ejaculation, which in clinical practice are often considered interchangeable phenomena.

A substantial overlap of ED with other forms of sexual dysfunction, including EjD and reduced sexual desire, as well as with other comorbid conditions, such as cardiovascular disease, diabetes, LUTS, and neurological disorders, has also been reported in previous studies [24,25,39,40]. Among men having ED with or without diabetes, those with EjD and/or reduced libido tended to have more severe ED [24,25]. Although ED, EjD, and OD are often influenced by psychogenic factors (e.g. anxiety, intercourse frequency), ejaculatory and orgasmic disorders (and reduced sexual desire) are more frequently (vs ED) ascribed to endocrinopathies (e.g. hypogonadism, hyperparathyroidism, hypothyroidism), serotonin receptor dysfunction, and genetic predispositions [36,39–41]. For endocrine factors, changes in neuroactive steroids (which have a range of potential central neuromodulatory effects) have been observed after ejaculation in young men, and the neuropeptide oxytocin is thought to be an important factor in physiological male ejaculatory function [42–45].

Also consistent with previous findings was our present observation that a history of antidepressant use was significantly associated with EjD and OD [46]. The pathological contribution of SSRIs to EjD is consistent with inhibitory effects of descending serotonergic neurones from the brainstem on ejaculatory responses. On the other hand, certain antipsychotics may blunt sexual responses via effects on central dopamine or prolactin, which are released

after male orgasm and may help to attenuate sexual desire during the post-ejaculatory refractory period [47–49]. The net effects of dopamine on EjF appear to be dose dependent, with a low dose of nonselective dopamine agonists inhibiting copulation (decreasing ejaculation frequency and intromission ratio) and a higher dose reducing ejaculation latency [50]. Antipsychotic therapies (particularly certain conventional neuroleptics) have also been associated with qualitative changes in orgasm, including dyspareunia [51]. Finally, treatment with certain α1-selective adrenoceptor blockers (for LUTS) has been associated with reduced ejaculate or expulsion force, possibly secondary to retrograde seminal flow into the bladder or inadequate contractions of pelvic-floor muscles or seminal vesicles [52–56].

POTENTIAL STUDY LIMITATIONS

The present form of *post hoc* associational research is of an inherently exploratory, hypothesis-generating nature. Because data were not adjusted statistically for multiple comparisons, some significant associations might be expected based on random probability alone. Further, our operational definitions of EjD and OD were somewhat arbitrary and not validated via objective measures (e.g. dynamic pelvic ultrasonography, functional MRI) or in large numbers of more heterogeneous male patient populations. Observational (non-interventional), associational research of the type conducted herein is inherently subject to potential biases and cannot determine the existence or direction of cause-effect relationships. For instance, do users of antidepressants have increased RRs of EjD or OD in isolation, or are these associations actually secondary to more severe ED in these settings? The present study also could not account for the effects of uncontrolled variables that might predispose a man with ED to EjD and/or OD. Responder bias may also have been present, in that most of the base tadalafil studies required men to make ≥4 attempts at sexual intercourse during the same 4-week treatment run-in period during which they reported their own EjF and OF on the IIEF. This requirement may have biased some men against reporting EjF or OF to be randomized to treatment for their ED, potentially resulting in underestimations of the degrees of EjD or OD in these individuals. The present study assessed EjD symptoms related to reduced or lack of ejaculate via IIEF-Q9. We did not assess other symptoms related to decreased force of ejaculation, painful ejaculation, or other aspects of EjD. Therefore, there may have been more subjects with EjD than reported herein. Although PE is studied extensively as a sexual dysfunction apart from ED [17,29,57–59], the present study would construe PE as normal EjF (because of the ways in which EjD and OD were defined). Hence, the present findings may not be generalizable to men with PE and with various forms of sexual dysfunction after treatment (our study included baseline factors only). The present findings warrant corroboration in prospective, controlled studies involving larger and more heterogeneous patient populations.

In summary, EjD and OD are common even in men with mild ED. However, the likelihoods of these conditions increase across rising strata of ED severity. Taken together, these findings suggest that male sexual dysfunction transcends ED, yet ED also remains a central impediment to healthy sexual function. Asian race; cardiomyopathy; and treatment with antidepressants or antipsychotics were significantly associated with increased RRs of EjD and OD. Further clinical studies are warranted to better understand associations between and among ED, EjD, and OD in men and the potential effects of these disorders on quality of life and of heterosexual partnerships.

ACKNOWLEDGEMENTS

Assistance in manuscript preparation was provided by Judith Daniels, PhD, and Stephen W. Gutkin, Rete Biomedical Communications Corp. (Wyckoff, NJ, USA), with support from the study sponsor.

CONFLICT OF INTEREST

This study and its report were supported by Eli Lilly and Company (Indianapolis, IN, USA). Darius A. Paduch and Alexander Bolyakov are Paid Investigators and/or Consultants/Advisors/Speakers for the study sponsor. Anthony Beardsworth and Steven D. Watts are employees of, and minor shareholders in, the study sponsor.

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Abbreviations: **PDE5**, phosphodiesterase type 5; **Ej(D)(F)**, ejaculatory (dysfunction) (function); **O(D)(F)**, orgasmic (dysfunction) (function); **E(D)(F)**, erectile (dysfunction) (function); **IIEF**, the International Index of Erectile Function; **PE**, premature ejaculation; **RR**, relative risk; **SSRI**, selective serotonin reuptake inhibitor; **RCTs**, randomized controlled trials.

BJUI BJU INTERNATIONAL

Nerve sparing can preserve orgasmic function in most men after robotic-assisted laparoscopic radical prostatectomy

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Accepted for publication 16 March 2011

Study Type – Therapy (case series)
Level of Evidence 4

OBJECTIVE

- To investigate orgasmic outcomes in patients undergoing robotic-assisted laparoscopic radical prostatectomy (RALP) and the effects of age and nerve sparing on these outcomes.

PATIENTS AND METHODS

- Between January 2005 and June 2007, 708 patients underwent RALP at our institution.
- We analysed postoperative potency and orgasmic outcomes in the 408 men, of the 708, who were potent, able to achieve orgasm preoperatively and available for follow-up.

RESULTS

- Of men aged ≤60 years, 88.4% (198/224) were able to achieve orgasm postoperatively in comparison to 82.6% (152/184) of older men ($P < 0.001$).
- Of patients who received bilateral nerve sparing (BNS) during surgery, 273/301 (90.7%) were able to achieve orgasm postoperatively compared with 46/56 (82.1%) patients who received unilateral nerve sparing and 31/51 (60.8%) men who received non-nerve-sparing surgery ($P < 0.001$).
- In men ≤60 years who also underwent BNS, decreased sensation of orgasm was present in 3.2% of men, and postoperative orgasmic rates were significantly better than men ≤60 years who underwent unilateral or no nerve sparing (92.9% vs 83.3% vs 65.4%, respectively; $P < 0.001$).
- Potency rates were also significantly higher in men ≤60 years and in those who underwent BNS.

CONCLUSIONS

- Age and nerve sparing influence recovery of orgasm and erectile function after RALP.
- Men ≤60 years old and those who undergo BNS are most likely to maintain normal sexual function.

KEYWORDS

cancer, robotic, prostatectomy, orgasmic, potency, outcomes, nerve sparing

What's known on the subject? and What does the study add?
Orgasm has a major influence on patients' satisfaction with the overall sexual experience, and alternations in orgasm are associated with significant reductions in emotional and physical satisfaction, which in turn may lead to sexual avoidance behaviour, disharmonious relationships and relationship breakdowns. Studies have found a reduction in orgasmic function after retropublic radical prostatectomy. While open radical prostatectomy inevitably damages some pelvic neuronal circuitry, which will thus impact on orgasmic responses, there is a paucity of data investigating the effect on robotic assisted radical prostatectomy on this.

To our knowledge this study represents the largest analysis of orgasmic function in the robotic prostatectomy literature, and therefore would be of value to surgeons in counseling candidates for RALP about orgasmic outcomes. In our series, young men (age ≤60 years) and those who underwent bilateral nerve sparing approaches had a better recovery of their premorbid orgasmic function when compared to older men or men with no nerve sparing.

INTRODUCTION

Prostate cancer is the commonest non-dermatological cancer affecting men in the Western world [1]. In the current PSA era there has been a demographic shift towards earlier detection of organ-confined prostate cancer at a younger age [2]. Radical prostatectomy (RP) remains the most common treatment for localized prostate cancer [3]. Although various surgical modifications and approaches, including nerve-sparing techniques, are currently available [4,5], sexual dysfunction (erectile and orgasmic) still continues to be a significant functional complication for many patients [6,7]. Most of the contemporary literature on sexual health outcomes after RP has been focused on erectile dysfunction rather than orgasmic responses.

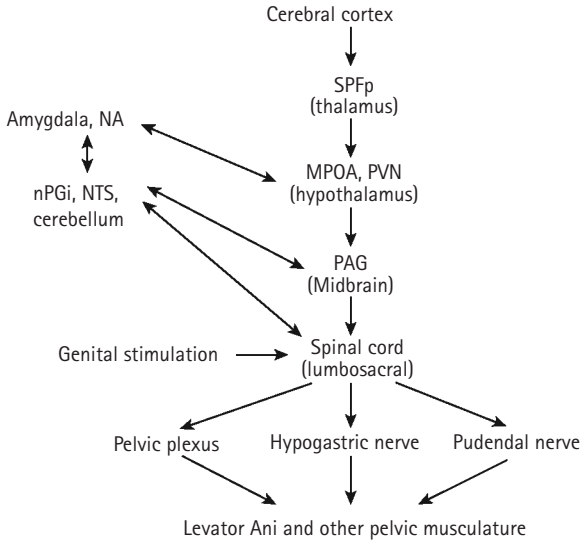


FIG. 1.
Neuronal pathway for orgasm. MPOA, medical preoptic area; NA, nucleus accumbens; nPGi, nucleus paragigantocellularis; NTS, nucleus tractus solitarii; PAG, periaqueductal gray; PVN, paraventricular nucleus of the hypothalamus; SPFp, subparafascicular nucleus of the thalamus.

Orgasm is associated with a series of regular sphincteric contractions of the bulbocavernosus muscle with an abrupt onset and termination [8]. While subjective perception of orgasm is associated with the contraction of the bulbocavernosus muscle, and orgasm typically occurs with ejaculation, neurophysiologically, orgasm and ejaculation are regulated by different neurotransmitters. Ejaculation is not necessary for orgasm as is evident in men who have undergone prostatectomy but who have normal orgasm [9]. What is known is that orgasm is mediated by complex interactions between autonomic and somatic nervous systems in conjunction with spinal cord circuits and higher brain centres [10] (Fig. 1). Arousal, defined as a progressive increase in the sensation of pleasure, results in progressive changes in neurotransmitters. Once the threshold level for orgasm is achieved an intense sensation of pleasure follows. The main regions involved in the feeling of reward that forms the subjective orgasmic response are the cortex, subparafascicular thalamic nucleus, amygdala and nucleus accumbens); the other regions illustrated in Fig. 1 are responsible mostly for the physical event of ejaculation. The pelvic (inferior hypogastric) plexus and pudendal and hypogastric nerves relay the afferent and efferent information during orgasm [10]. Normal integration of tactile, olfactory, visual and auditory sexual cues during arousal is necessary for orgasm to occur, but it is unclear to what extent the afferent signalling from the pelvic muscles and open nerve endings on the penis contribute to progression of arousal and orgasm. The fact that some men can achieve orgasm without tactile stimulation to the genital region (through auditory or visual cues) clearly indicates that orgasm is at least partly central and not a purely peripheral event.

Orgasm has a major influence on patients' satisfaction with the overall sexual experience [11], and alterations in orgasm are associated with significant reductions in emotional and physical satisfaction, which in turn may lead to sexual-avoidance behaviour, disharmonious relationships and relationship breakdowns [12]. One notable study investigated orgasmic function after retropubic RP and showed that only 22% of patients reported no change in their premorbid state [13]. Although open RP inevitably damages some pelvic neuronal circuitry, which will impact on orgasmic responses, there is a paucity of data investigating the effect of robotic-assisted laparoscopic radical prostatectomy (RALP) on this. Hence, we investigated the effect of RALP on orgasmic function.

PATIENTS AND METHODS

Between January 2005 and June 2007, 708 patients underwent RALP at our institution by a single surgeon (AT). Patients who were both potent (International Index of Erectile Function ≥60) and able to achieve orgasm preoperatively were eligible for this study. Orgasmic function was defined preoperatively using a physician-reported binary scale (yes/no on direct questioning of the patient). In all, 444 patients fulfilled our eligibility criteria and gave informed consent, of whom 36 patients were lost to follow-up; as a result, the final study cohort consisted of 408 patients. We analysed orgasmic and erectile outcomes in these patients as part of our Institutional Review Board-approved quality of life study. During evaluation in the clinical office before surgery, patients provided demographic information and completed self-administered standardized health-related quality of life (HRQOL) questionnaires, including the Expanded Prostate Cancer Index Composite (EPIC), and the International Index of Erectile Function. Outcomes questionnaires containing items from the sexual function domain of the EPIC HRQOL were dispatched to patients via postal or electronic mail at regular intervals after their surgery. Specifically, subjects were asked to evaluate their postoperative orgasmic function into one of five categories: normal, diminished, absent, better, or early. They were also asked to comment on whether orgasm was painful and their level of satisfaction with their orgasmic function. Subjects who did not respond to the questionnaire were then contacted via telephone by a member of the research team to ensure receipt of the questionnaire. Patients were considered potent when they achieved erections sufficient for vaginal intercourse. Additional data regarding satisfaction and pain during orgasm, as well as use of phosphodiesterase type 5 inhibitors (PDE5i) were obtained. Data collection and follow-up correspondence were performed in compliance with the Health Insurance Portability and Accountability Act.

The detailed preoperative clinicopathological characteristics of the cohort are shown in Table 1. Mean follow-up was 36 months in our cohort (median 35 months; range 24–53 months). We further stratified our cohort based on age and nerve sparing (Fig. 2). We also compared orgasmic outcomes in men who were potent and those who were impotent after surgery. Apart from the data obtained from the follow-up questionnaire, preoperative clinical data such as PSA, clinical stage and biopsy Gleason score were abstracted from medical records. Intraoperative notes were reviewed to obtain nerve-sparing data. The returned responses

to the outcomes questionnaires, along with the patients' preoperative, operative and postoperative clinicopathological data were prospectively entered into an Institutional Review Board-approved password-protected MICROSOFT® ACCESS database.

Statistical analysis was performed using PASW v18.0 (SPSS Inc., Chicago, IL, USA), with statistical significance considered at $P < 0.05$. Chi-squared test was used to evaluate the impact of age and nerve sparing on orgasmic function and potency after RALP.

RESULTS

In all, 198/224 (88.4%) men aged ≤60 years and 152/184 (82.6%) aged >60 years were able to achieve orgasm postoperatively. Postoperatively, potency was present in 190/224 (84.8%) men aged ≤60 years and in 142/184 (77.1%) men aged >60 years. Postoperative sexual function outcomes, for both orgasm and potency, were significantly ($P < 0.001$) better in men ≤60 years compared with older men (Table 2).

Postoperatively, 273 (90.7%) men with bilateral nerve sparing, 46 (82.1%) men with unilateral nerve sparing and 31 (60.8%) men with no nerve sparing were able to achieve orgasm ($P < 0.001$). Postoperative potency rates were also significantly higher in men with bilateral nerve sparing (86.7%) when compared with the unilateral nerve-sparing (71.4%) and non-nerve-sparing (60.8%) groups ($P < 0.001$) (Table 3).

In men ≤60 years of age, a significantly higher percentage of patients who underwent bilateral nerve sparing had orgasm postoperatively (92.9% vs 83.3% vs 65.4%, $P < 0.001$) and regained potency (90.5% vs 73.3% vs 61.5%; $P < 0.001$) when compared with patients who had unilateral nerve-sparing and non-nerve-sparing surgery, respectively (Table 4, Fig. 3).

Of the men who had postoperative potency, most had orgasm as well (79.5% of those <60 years; 83.1% of those who underwent bilateral nerve sparing, and 84.5% of those <60 years who also underwent bilateral nerve sparing). However, the rates of orgasm were much lower in men who were not potent after surgery (5.4% of those <60 years; 3.7% of those who underwent

Variable	Number of patients ($N = 408$)
Age, median (IQR)	60 (55,65)
60 years or less, *n* (%)	224 (54.9)
More than 60 years, *n* (%)	184 (45.1)
BMI, median (IQR)	26 (24, 29)
Preoperative IIEF, median (IQR)	69 (65, 72)
Preoperative PSA, median (IQR)	5 (3.9,6.6)
Clinical stage, *n* (%)	
T1	349 (85.5)
T2	59 (14.5)
Biopsy Gleason, *n* (%)	
≤6	166 (40.7)
7 (3 + 4)	179 (43.9)
7 (4 + 3)	38 (9.3)
≥8	25 (6.1)
Prostate volume, median (IQR)	45.5 (37.3, 54.3)
Pathology Gleason, *n* (%)	
≤6	167 (40.9)
7 (3 + 4)	182 (44.6)
7 (4 + 3)	36 (8.8)
≥8	23 (5.7)
Pathology stage, *n* (%)	
T2	349 (85.5)
T3	59 (14.5)
Positive surgical margin, *n* (%)	34 (8.3)

TABLE 1 Preoperative variables, baseline demographics, biopsy and pathologic data of the cohort

BMI, body mass index; IIEF, International Index of Erectile Function; IQR, interquartile range; PSA, prostate-specific antigen.

TABLE 2 Postoperative sexual function outcomes with relation to age

	Age ≤60 years ($N = 224$), *n*/*N* (%)	Age >60 years ($N = 184$), *n*/*N* (%)	*P* value
Orgasmic outcomes			<0.001
Postoperative orgasm present	198/224 (88.4)	152/184 (82.6)	
Have same orgasm	180/224 (80.3)	147/184(80)	
Diminished orgasm	13/224(5.8)	3/184 (1.6)	
Better orgasm	4/224 (1.8)	1/184(0.5)	
Early orgasm	1/224 (0.5)	1/184 (0.5)	
Postoperative orgasm absent	26/224 (11.6)	32/184 (17.4)	
Potency outcomes			<0.001
Postoperative potent	190/224 (84.8)	142/184 (77.1)	
Postoperative potent with bilateral nerve sparing	152/168 (90.5)	109/133 (82)	

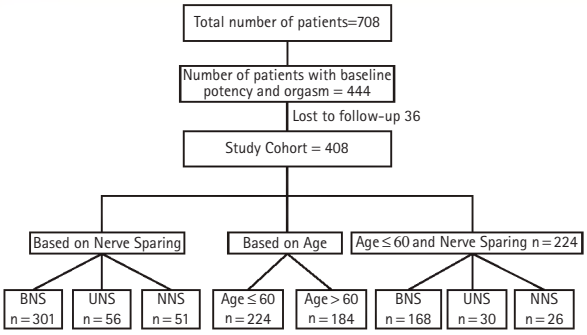


FIG. 2. Characteristics of the study cohort. BNS, bilateral nerve sparing; UNS, unilateral nerve sparing; NNS, non-nerve sparing.

TABLE 3 Postoperative sexual function outcomes with relation to nerve sparing

	BNS (*N* = 301), *n*/*N* (%)	UNS (*N* = 56), *n*/*N* (%)	NNS* (*N* = 51), *n*/*N* (%)	*P* value
Orgasmic outcomes				<0.001
Postoperative orgasm present	273/301 (90.7)	46/56 (82.1)	31/51 (60.8)	
Have same orgasm	257/301 (85.4)	42/56 (75)	28/51 (54.9)	
Diminished orgasm	12/301 (4)	2/56 (3.5)	2/51 (3.9)	
Better orgasm	3/301 (1)	1/56 (1.8)	1/51 (2)	
Early orgasm	1/301 (0.3)	1/56 (1.8)	0	
Postoperative orgasm absent	28/301 (9.3)	10/56 (17.9)	20/51 (39.2)	
Potency outcomes				<0.001
Postoperative potent	261/301 (86.7)	40/56 (71.4)	31/51 (60.8)	
Postoperative potent if age ≤60 years	152/168 (90.5)	22/30 (73.3)	16/26 (61.5)	

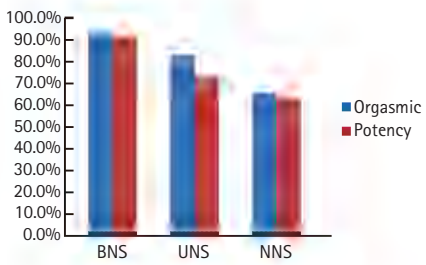
BNS, bilateral nerve sparing; UNS, unilateral nerve sparing; NNS, non-nerve sparing.
**Includes patients with incremental nerve sparing and nerve reconstruction.*

TABLE 4 Postoperative sexual function outcomes in patients age ≤60 years and nerve sparing

	BNS (*N* = 168), *n*/*N* (%)	UNS (*N* = 30), *n*/*N* (%)	NNS* (*N* = 26), *n*/*N* (%)	*P* value
Orgasmic outcomes				<0.001
Postoperative orgasm present	156/168 (92.9)	25/30 (83.3)	17/26 (65.4)	
Have same orgasm	144/168 (85.7)	22/30 (73.3)	14/26 (53.8)	
Diminished orgasm	9/168 (5.4)	2/30(6.7)	2/26 (7.7)	
Better orgasm	2/168 (1.2)	1/30 (3.3)	1/26 (3.9)	
Early orgasm	1/168(0.6)	0	0	
Postoperative orgasm absent	12/168 (7.1)	5/30 (6.7)	9/26 (34.6)	
Potency outcomes				<0.001
Postoperative potent	152/168 (90.5)	22/30 (73.3)	16/26 (61.5)	

BNS, bilateral nerve sparing; UNS, unilateral nerve sparing; NNS, non-nerve sparing.
**Includes patients with incremental nerve sparing and nerve reconstruction.*

FIG. 3. Postoperative potency and orgasmic outcomes in patients with age ≤60 years, in relation to nerve sparing. BNS, bilateral nerve sparing; UNS, unilateral nerve sparing; NNS, non-nerve sparing.



bilateral nerve sparing, and 6.0% of those <60 years who also underwent bilateral nerve sparing); these differences in orgasmic function were statistically significant between potent and impotent men.

From our questionnaire, we also calculated satisfaction rates (using a rating of very low, low, moderate, high, or very high) and pain associated with orgasm in patients with age ≤60 years and bilateral nerve sparing. Out of 156 patients who had postoperative orgasm, 82% had high or very high, 10.2% had moderate, 7.1% had very low or low satisfaction rates associated with orgasm. Only 3.2% (5/156) of patients complained of pain associated with orgasm. Use of PDE5i did not affect return of orgasmic function with similar usage rates in those with and without orgasm (61% vs 65%, respectively).

DISCUSSION

Orgasm is a compelling, brief event that is an integration of cognitive, emotional, somatic, visceral and neural processes [14]. It is a combination of physiological and psychological changes that occur coincident with ejaculation in men. Physiological changes during orgasm are tachycardia, sweating, muscle contraction and rhythmic contraction of the ejaculatory apparatus [15], whereas psychological changes include an altered state of consciousness, release of tension and emotional euphoria. As the ejaculatory apparatus (prostate, seminal vesicles and ejaculatory ducts) is removed at RP, the patients subsequently cannot emit sperm and so have 'dry ejaculations' [16]. Other changes in orgasm such as decreased intensity, anorgasmia and dysorgasmia have also been reported in patients after RP [16–18]. The consistency, quality and satisfaction of orgasm can significantly affect quality of life [16,17,19]. Therefore, any alteration in orgasm, especially its absence, is associated with significant reductions in emotional and physical satisfaction, which may in turn lead to avoidance of sexual activity and disharmony in intimate relationships [12].

Although ejaculation is coincident with orgasm in many men, the subjective sensation of orgasm can occur without ejaculation. The pelvic plexus, pudendal nerve and hypogastric nerve are involved in both sensory and motor innervations of orgasm, such that orgasm is mediated by the sympathetic, parasympathetic and somatic nervous systems. The pelvic plexus lies within a fibro-fatty plate that is flat, rectangular, sub-peritoneal, sagittal and symmetrical. It arises at the level of the intersection between the vas deferens and the terminal pelvic ureter and follows the postero-lateral aspect and circumvolutions of the seminal vesicle, with which there is a plane of surgical cleavage [20]. Our technique of posterior neural preservation in which we take great care to preserve the pelvic plexus plus its downstream fibres that form the proximal neurovascular plate, predominant neurovascular bundle, and accessory distal neural pathways [21] may therefore be responsible for our improved postoperative orgasmic rates with nerve sparing compared with non-nerve sparing. It may also be possible that some men have more damage to the bulbocavernosus muscle and the distal fibres of the pudendal nerve during non-nerve sparing as a result of more aggressive dissection generally, accounting for the lack of orgasm. In our series, young men (age ≤60 years) had

improved postoperative orgasmic function when compared with older men. Earlier series have also reported age-dependent responses, where younger patients did well postoperatively both in terms of orgasm and erectile function [18]. Hypogonadism and penile hyposensitivity also increase with age and this may also account for why orgasm is diminished in older patients. Hollenbeck *et al.* [22] used the EPIC validated questionnaire to study a cohort of 671 patients undergoing open RP. They reported that nerve-sparing technique, patient age, prostate size, time since prostatectomy, income and education level were significant independent predictors of sexual health outcomes after prostatectomy. Patients below 58 years of age were able to achieve orgasm in 84%, 68% and 67% after bilateral, unilateral and non-nerve-sparing surgery respectively compared with patients with aged over 69 years who achieved orgasm in 58%, 58% and 30%, respectively.

Van der Aa *et al.* [23] studied the effect of unilateral nerve-sparing surgery on orgasm and reported that 84.8% of men had the ability to achieve orgasm postoperatively whereas erectile function recovery was 45% for partial erections and 30% for complete recovery at 18 months. In our series we found that 90.7% and 82.1% of men were able to achieve orgasm with bilateral and unilateral nerve sparing, respectively, after a follow-up of at least 12 months. Noldus *et al.* [24] found that 29% of men with unilateral nerve sparing and 52% men with bilateral nerve sparing were potent postoperatively. Further, about 80% of men had unchanged, 9% had improved and 11% had decreased experience of orgasmic function irrespective of whether they were potent or not. Our own results, however, seem to suggest that orgasmic function is closely allied to potency. Men who were potent postoperatively were far more likely to preserve orgasm than those who were not. It may be therefore that nerve sparing improves orgasm by its confounding effect on potency.

The cause of dysorgasmia is not well understood. It is postulated that the physiological bladder neck closure that occurs during orgasm in men after an RP translates into spasm of the vesico-urethral anastomosis or pelvic floor musculature dystonia [25]. In this study we found that dysorgasmia was present in 3% patients aged ≤60 who underwent bilateral nerve sparing and had a follow-up of at least 12 months. Similar results have been shown in previous studies. Barnas *et al.* [13] in their retrospective study found that 14% of the patients had dysorgasmia. In these men 33% experienced pain always (i.e. with every orgasm), frequently in 13%, occasionally in 35% and rarely in 19%. Most patients (55%) had orgasm-associated pain of less than 1-min duration.

In this study we did not find any significant improvement in orgasmic function with the use of PDE5i. The literature regarding the effect of PDE5i on orgasmic function after RP is scarce, although one group confirmed our findings [26].

This study has a number of limitations. We did not assess orgasm-associated urine leakage (climacturia) in these patients, and this would be a useful parameter to measure because there is good evidence that this impacts on sexual satisfaction levels [27]. Other orgasm-related symptoms were also not evaluated: orgasmic headaches, epileptiform aura or migraines triggered by orgasm and male multiple orgasms. However, all of these are rare and so unlikely to have significantly confounded our findings. Also, our assessment of orgasm was purely subjective and defined by the patient; we made no attempt to validate the accuracy of reporting normal, diminished, improved, early, absent, or painful orgasm using a neurophysiological approach. Investigators have shown that serum prolactin levels increase for over 1 hour after orgasm [28]. However, the subjective feeling of orgasm is what is most important for the patient so this is not a major limitation. Although we investigated the use of PDE5i and whether patients were able to orgasm or not, we did not record the use of other neuropharmacological agents that have been shown to affect climactic sensation: selective serotonin reuptake inhibitors, antidepressants, opiates, anticonvulsants and antipsychotics [29–32].

Another limitation is that we did not assess the psychological factors that could impact on orgasmic outcomes in our patients, and we did not ask patients whether they had sought counselling or other more intensive psychological therapies, all of which may confound our findings. Third, we did not look at earlier follow-up time-points so cannot comment on the effects of surgery on orgasmic function in the first postoperative year. Fourth, although our preoperative measure of erectile function used a validated assessment tool, the International Index of Erectile Function, our postoperative follow-up measure of potency was based on a single question: the ability or not to have an erection sufficient for penetrative vaginal intercourse. Of course this is likely to introduce bias in our results because there is evidence that this single-question approach is not as accurate as the validated International Index of Erectile Function [33–34]. However, we were consistent in our use of our assessment tools such that any differences we found between groups are still valid. Nevertheless, we cannot exclude the possibility that improved potency above and beyond the definition applied in the young and bilateral nerve-sparing groups confounded our improved orgasmic results. Also, while we found that men who were able to have vaginal intercourse (our definition of postoperative potency) were more likely to preserve orgasm, we did not measure potency or orgasmic function on a quantitative scale, and so cannot comment on whether highly potent men had improved orgasmic function compared with men who were only just able to sustain an erection hard enough for penetration. As potency function might be a confounding factor for orgasmic function, we are currently performing a prospective study looking at this issue. Finally, our follow-up period ranged from 24 to 53 months so there is the possibility that time itself might have confounded our results. However, most functional outcomes have stabilized by 1 year postoperatively so this is unlikely to have changed the findings significantly. Despite these limitations in our dataset, to our knowledge this study represents the largest analysis of orgasmic function in the robotic prostatectomy literature, and we feel it would be of value to surgeons when counselling candidates for RALP about orgasmic outcomes.

Orgasmic dysfunction is a common complication associated with radical prostatectomy. It can take the form of decreased intensity, absence or dysorgasmia. Younger men and those who undergo bilateral nerve-sparing approaches are most likely to recover their premorbid orgasmic function.

CONFLICT OF INTEREST

Dr Ashutosh Tewari has received research grants from the Intuitive Surgical and the Prostate Cancer Foundation; he is also the endowed Ronald P. Lynch Professor of Urologic Oncology and Director of the Lefrak Center of Robotic Surgery and Prostate Cancer Institute, Weill Cornell Medical College. Dr Prasanna Sooriakumaran is the ACMI Corp. Endourological Society Corporate Fellow and also receives financial sponsorship from Prostate UK.

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Abbreviations: **RP**, radical prostatectomy; **PDE5i**, phosphodiesterase type 5 inhibitor.

Effects of 12 weeks of tadalafil treatment on ejaculatory and orgasmic dysfunction and sexual satisfaction in patients with mild to severe erectile dysfunction: integrated analysis of 17 placebo-controlled studies[1]

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[1]Selected data were presented at the 14th Congress of the European Society for Sexual Medicine, 1–4 December 2011, Milan, Italy.

What's known on the subject? and What does the study add?

- Disorders of ejaculation and orgasm are common, even in men with only mild erectile dysfunction (ED).
- Treatment with the phosphodiesterase type-5 inhibitor tadalafil was associated with improvements in ejaculatory and orgasmic function. Patients with residual ejaculatory or orgasmic dysfunction experience reduced sexual satisfaction. These findings need to be corroborated in further clinical trials involving men without ED.

Objectives

- To compare effects of tadalafil on ejaculatory and orgasmic function in patients presenting with erectile dysfunction (ED).
- To determine the effects of post-treatment ejaculatory dysfunction (EjD) and orgasmic dysfunction (OD) on measures of sexual satisfaction.

Patients and Methods

- Data from 17 placebo-controlled 12-week trials of tadalafil (5, 10, 20 mg) as needed in patients with ED were integrated.
- EjD and OD severities were defined by patient responses to the International Index of Erectile Function, question 9 (IIEF-Q9; ejaculation) and IIEF-Q10 (orgasm), respectively.
- Satisfaction was evaluated using the intercourse and overall satisfaction domains of the IIEF and Sexual Encounter Profile question 5.
- Analyses of covariance were performed to compare mean ejaculatory function and orgasmic function, and chi-squared tests evaluated differences in endpoint responses to IIEF-Q9 and IIEF-Q10.

Results

- A total of 3581 randomized subjects were studied.
- Treatment with tadalafil 10 or 20 mg was associated with significant increases in ejaculatory and orgasmic function (vs placebo) across all baseline ED, EjD, and OD severity strata.
- In the tadalafil group, 66% of subjects with severe EjD reported improved ejaculatory function compared with 36% in the placebo group ($P < 0.001$).
- Similarly, 66% of the tadalafil-treated subjects (vs 35% for placebo; $P < 0.001$) with severe OD reported improvement.
- Residual severe EjD and OD after treatment had negative impacts on sexual satisfaction.
- Limitations of the analysis include its retrospective nature and the use of an instrument (IIEF) with as yet unknown performance in measuring treatment responses for EjD and OD.

Conclusions

- Tadalafil treatment was associated with significant improvements in ejaculatory function, orgasmic function and sexual satisfaction.
- Proportions of subjects reporting improved ejaculatory or orgasmic function were ≈ twofold higher with tadalafil than with placebo.
- These findings warrant corroboration in prospective trials of patients with EjD or OD (without ED).

Keywords

ejaculation, epidemiology, erectile dysfunction, human, male, orgasm, phosphodiesterases, tadalafil

Introduction

Disorders of ejaculation (ejaculatory dysfunction [EjD]) and orgasm (orgasmic dysfunction [OD]) are frequent and bothersome yet under-reported male sexual difficulties [1–8]. Conversely, sexual satisfaction is positively correlated with the likelihood of orgasm in both men and women [9,10]. Ejaculation is defined as expulsion of semen from the urethra. Orgasm is a subjective sensation of intense pleasure which typically, but not always, is associated with ejaculation. EjD includes a range of disorders such as premature ejaculation (PE), delayed ejaculation (DE), decreased volume and force of ejaculation, as well as inability to ejaculate (anejaculation). Inability to achieve orgasm (anorgasmia), regardless of the presence of ejaculation, or decreased intensity of pleasure (hyporgasmia) constitute disorders of orgasm.

Delayed ejaculation (prevalence = 0–11%) [5,7,11–14] is often highly bothersome. In the Multinational Survey of the Aging Male (MSAM-7), 46% of respondents reported reduced semen volume, 5% reported anejaculation, and 7% reported pain/discomfort on ejaculation [2]. In our previous study, we showed that 15.6% of men reported poor orgasmic sensation despite normal ejaculatory function [15]. A frequent absence of orgasm can result in relationship discord secondary to decreased physical and emotional satisfaction in men and their sexual partners [9,16,17]. Like erectile dysfunction (ED), both EjD and OD are potential sources of psychological distress, embarrassment or even shame for the male patient.

Phosphodiesterase type-5 (PDE-5) inhibitors have been evaluated as potential treatments for EjD, particularly PE [18–24]. However, much less is known about the effects of PDE-5 inhibitors on other forms of EjD and OD. A small open-label study including 30 male patients with infertility and sexual dysfunction (both PE and DE) showed improved ejaculatory function with sildenafil treatment [25].

Our group recently reported findings from a retrospective, integrated analysis of baseline data among >12 000 male subjects in clinical trials enrolling patients with ED [15]. The analysis showed that only 36% of subjects with ED reported normal orgasmic function and 42% reported normal ejaculatory function. Although OD and EjD were increasingly likely across advancing ED severity strata, both conditions were common complaints, even in men with mild ED. The objective of the current analysis was to investigate the effects of 12 weeks of tadalafil treatment (vs placebo) on EjD and OD as well as sexual satisfaction in patients enrolled in ED trials.

Trial Registration

Internal study identifiers with available ClinicalTrials.gov registration numbers were Study H6D-MC-LVDI (ClinicalTrials.gov identifier NCT00547495) and H6D-MC-LVDY (ClinicalTrials.gov identifier NCT00547573) (http://www.clinicaltrials.gov).

Patients and Methods

Details concerning study designs and populations in these trials have been published elsewhere, including in previous pooled-data analyses [26]. All 17 studies were double-blind and evaluated the clinical efficacy of tadalafil for ED at baseline and every 4 weeks; primary endpoints for all studies were based on efficacy data collected at the 12-week visit. These trials evaluated as-needed dosing of tadalafil 5, 10 and 20 mg. Subjects were instructed to attempt coitus at least four times each month for the duration of the clinical trial.

There is no validated instrument to measure diverse dimensions of EjD and OD. As a result, in this exploratory study we used subject-reported ability to ejaculate ('how often you ejaculated') through intravaginal intercourse (question 9 [Q9]) and subject-reported feeling of orgasm ('how often you felt orgasm regardless of the presence or lack of ejaculation') (Q10) of the International Index of Erectile Function (IIEF) questionnaire [27]. The IIEF was administered at each study visit, and a Sexual Encounter Profile (SEP) diary was completed by subjects after each sexual attempt. Table 1 summarizes the ways in which responses were categorized for analysis purposes.

Sexual satisfaction was assessed using both the intercourse satisfaction and overall satisfaction domains of the IIEF (i.e.

Table 1 Categorization of patient responses IIEF-Q9* and IIEF-Q10†.

IIEF response	How categorized (severity of EjD or OD)
No intercourse attempts	Not analysed
Never/almost never	Severe
Sometimes/about half the time	Moderate
Always/almost always	Mild/no dysfunction

*IIEF-Q9: 'Over the past 4 weeks, when you had sexual stimulation or intercourse, how often did you ejaculate?' †IIEF-Q10: 'Over the past 4 weeks, when you had sexual stimulation or intercourse, how often did you have the feeling of orgasm with or without ejaculation?'

IIEF-IS and IIEF-OS, respectively) as well as SEP question 5 (SEP5: 'Were you satisfied overall with this sexual experience?').

Central endpoints comprised mean changes from baseline in scores on the IIEF-Q9, IIEF-Q10, mean IIEF-IS, IIEF-OS and SEP5, as well as distributions of responses to IIEF-Q9 and IIEF-Q10 after 12 weeks of treatment for subjects with severe EjD or OD at baseline. For these patients with severe EjD or OD, endpoint responses of 1 or 2 to IIEF-Q9 or IIEF-Q10 were operationally defined as 'no improvement', whereas 'improvement' was defined as an endpoint IIEF-Q9 or IIEF-Q10 response of 3, 4 or 5.

The study population included all patients who were randomized and started study medication. For patients who discontinued, endpoint data were derived using the last-observation-carried-forward (LOCF) imputation rule. If, for a particular analysis and study patient, no post-baseline data were collected, that patient was not included in the analysis. Subjects with IIEF-Q9 or IIEF-Q10 responses of '0' (no sexual activity) were not included in the corresponding analyses.

Analyses of covariance (ANCOVA) models with effects for study, baseline and treatment group were used to evaluate differences in mean changes. Chi-squared tests were conducted to evaluate differences in categorical responses. All statistical tests were two-sided at $\alpha = 0.05$. All analyses performed to support this disclosure were of a *post hoc* nature and did not control for multiplicity.

Results

A total of 3581 subjects were randomized, including 1512 (42%) with severe EjD and 1812 (51%) with severe OD. The distributions of baseline EjD and OD severity were balanced across treatment groups. The mean (SD) age of study subjects was 54.9 (11.3) years, and the mean (SD) body mass index was 26.8 (4.2) kg/m^2. Approximately half the population were Caucasian (50.9%) and 39.3% were of Asian descent (Table 2).

Treatment with tadalafil was associated with a significant increase in ejaculatory function (vs placebo), as measured using the IIEF-Q9 (Fig. 1). In patients with severe EjD at baseline, treatment with tadalafil was associated with a significant least-squares (LS) mean increase in IIEF-Q9, in a dose-related manner: 1.6, 1.9 and 2.0 for tadalafil 5, 10 and 20 mg, respectively. There was a significant improvement in IIEF-Q9 in patients with moderate baseline EjD taking tadalafil 20 mg. In patients with minimal or no EjD at baseline, tadalafil treatment was associated with a reduced worsening of EjD compared with placebo (Fig. 1).

Likewise, significant LS mean increases in orgasmic function (vs placebo) were associated with tadalafil treatment (Fig. 2). In patients with severe OD at baseline, treatment with tadalafil was associated with a significant increase in the mean IIEF-Q10 score, in a dose-related manner: 1.3, 1.8 and 2.0 for tadalafil 5, 10 and 20 mg, respectively. Significant (but non-dose-related) improvements in patients with moderate OD, and

Table 2 Baseline demographic and clinical characteristics of all randomized subjects receiving placebo (or tadalafil 5, 10 or 20 mg) as needed. Data are presented as *n* (%) unless noted otherwise*.

Baseline characteristic	Tadalafil (*N* = 2579)	Placebo (*N* = 1002)	Total (*N* = 3581)
Mean (SD) age, years	54.8 (11.4)	55.0 (11.2)	54.9 (11.3)
Mean (SD) BMI, kg/m^2	26.8 (4.3)	26.7 (4.1)	26.8 (4.2)
Race			
Caucasian	1350 (52.3)	473 (47.2)	1823 (50.9)
African	65 (2.5)	16 (1.6)	81 (2.3)
Asian	965 (37.4)	443 (44.2)	1408 (39.3)
Other	199 (7.7)	70 (7.0)	269 (7.5)
Region			
North America	723 (28.0)	252 (25.1)	975 (27.2)
Europe	411 (15.9)	131 (13.1)	542 (15.1)
Asia	952 (36.9)	437 (43.6)	1389 (38.8)
Latin America	329 (12.8)	113 (11.3)	442 (12.3)
Middle East	71 (2.8)	22 (2.2)	93 (2.6)
Oceania (Australia)	93 (3.6)	47 (4.7)	140 (3.9)*
Comorbidities			
Diabetes mellitus	548 (21.2)	227 (22.7)	775 (21.6)
Hypertension	715 (27.7)	274 (27.3)	989 (27.6)
Hyperlipidaemia	318 (12.3)	149 (14.9)	467 (13.0)
Ischaemic disorders	154 (6.0)	72 (7.2)	226 (6.3)

**Some percentages do not add to 100 because of rounding.*
BMI, body mass index.

Fig. 1 Least-squares mean changes in ejaculatory function from baseline to study endpoint (week 12 or LOCF) among subjects with different degrees of baseline EjD as assessed using IIEF-Q9, 'Over the past 4 weeks, when you had sexual stimulation or intercourse, how often did you ejaculate?' Operational definitions of EjD were as follows: minimal/no EjD, a response of 4 to IIEF-Q9 ('most times/much more than half the time') or 5 ('almost always/always'); moderate EjD, a response of 3 ('sometimes/about half the time'); and severe EjD, a response of 1 ('almost never/never') or 2 ('a few times/much less than half the time'). *$P < 0.05$ vs placebo. Numbers above and below axes represent patients with available data.

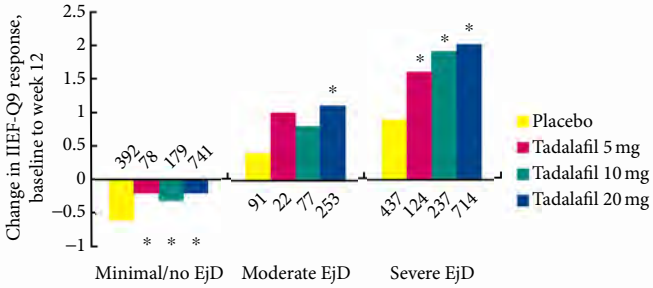
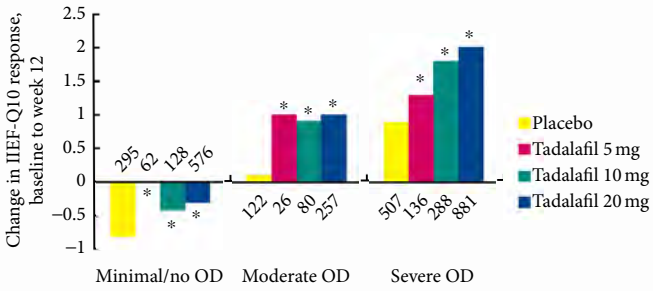


Fig. 2 Least-squares mean changes in orgasmic function from baseline to study endpoint (week 12 or LOCF) among subjects with different degrees of baseline OD as assessed using IIEF-Q10, 'Over the past 4 weeks, when you had sexual stimulation or intercourse, how often did you have the feeling of orgasm or climax?' Operational definitions of OD were as follows: severe OD, a response of 1 ('almost never/never') or 2 ('a few times/much less than half the time') to IIEF-Q10; moderate OD, a response of 3 ('sometimes/about half the time'); and minimal/no OD, a response of 4 ('most times/much more than half the time') or 5 ('almost always/always'). *$P < 0.05$ vs placebo. Numbers above and below the axes represent patients with available data.



significantly reduced worsening of orgasmic function in patients with minimal or no OD were also observed in tadalafil-treated groups (Fig. 2).

Irrespective of baseline ED severity, subjects randomized to each dose of tadalafil experienced significant improvement in ejaculatory and orgasmic function from baseline to study completion ($P < 0.05$ for each tadalafil dose vs placebo, except for the 5 mg dose for orgasmic function in patients with severe ED at baseline; Table 3).

Significantly higher proportions of subjects with severe baseline EjD or OD who received tadalafil experienced improvements compared with those on placebo: approximately two-thirds with active treatment compared with one-third with placebo (Table 4). In the tadalafil group, 66% of subjects with severe baseline EjD reported improvement, compared with 36% in the placebo group ($P < 0.001$). Corresponding data in subjects with severe baseline OD were 66% of tadalafil-treated subjects reporting improvement compared with 35% in the placebo group ($P < 0.001$).

Tadalafil treatment was associated with significant increases in both SEP and IIEF measures of sexual satisfaction (vs placebo) (Figs 3,4; Table 5). For instance, treatment with tadalafil 20 mg was associated with an LS mean increase in per-patient percent 'yes' responses to SEP5 of 19.2% among patients with severe EjD at study completion, 28.6% among those with moderate EjD, and 57.6% among those with mild or no EjD ($P < 0.001$ vs placebo for each). Corresponding data for OD after tadalafil 20 mg were LS mean increases of 20.5% among patients with severe OD at study completion, 30.5% among those with moderate OD, and 59.6% among those with minimal or no OD ($P < 0.001$ vs placebo for each).

To determine the differential effect of EjD and OD on responses to measures of sexual satisfaction, we compared mean scores across EjD and OD severity categories after 12 weeks of tadalafil therapy. In general, patients with severe EjD or OD who were treated with tadalafil 20 mg had endpoint means that were ≈ 20% lower than those of subjects with moderate EjD or OD on both the IIEF-IS and IIEF-OS domains. Similarly, patients with moderate EjD or OD had endpoint means that were ≈ 20% lower than those of patients with mild/no EjD or OD. Taken together, these consistent findings suggest that each increased degree of EjD or OD severity was associated with a 20% decrease in sexual satisfaction as measured by the IIEF-IS and IIEF-OS domains.

A similar evaluation of SEP5 responses suggested, for tadalafil 20 mg treatment groups (Figs 5,6), a decrease of ≈30–40% in per-patient sexual satisfaction for an increase from moderate to severe EjD or OD; a decrement of ≈50% in satisfaction rates for an increase from no/mild to moderate EjD or OD; and a decline of ≈65% in satisfaction rates for an increase from no/mild to severe EjD or OD. These results indicated that residual EjD and OD significantly impaired sexual satisfaction.

Tadalafil was well tolerated in the base studies. The most frequent treatment-emergent adverse events (e.g. headache, dyspepsia, back pain) have been reported in previous pooled-data analyses [26].

Table 3 Least-squares mean changes from baseline to study endpoint (week 12 or LOCF) in IIEF-Q9 and IIEF-Q10 by treatment group and baseline severity of ED.

Variable/subgroup	As-needed treatment group			
	Placebo	Tadalafil 5 mg	Tadalafil 10 mg	Tadalafil 20 mg
IIEF-Q9				
Baseline ED severity				
Mild/no (IIEF-EF* = 17–30)	0	0.7*	0.4*	0.6*
Moderate (IIEF-EF = 11–16)	0.3	0.8*	0.9*	1.1*
Severe (IIEF-EF = 1–10)	0.3	0.7*	1.2*	1.5*
IIEF-Q10				
Baseline ED severity				
Mild/no (IIEF-EF = 17–30)	0	0.8*	0.6*	0.7*
Moderate (IIEF-EF = 11–16)	0.2	0.9*	1.2*	1.2*
Severe (IIEF-EF = 1–10)	0.3	0.7	1.2*	1.5*

*$P < 0.05$ vs placebo.
EF, erectile function domain.

Table 4 Distribution of 12-week responses to IIEF-Q9* and IIEF-Q10† in subjects with severe baseline EjD or OD by treatment group. Data are presented as *n* (%) unless noted otherwise.

Variable	Treatment (*N*)	Improvement (IIEF response)‡	
		No (1 or 2)	Yes (3, 4 or 5)
IIEF-Q9: ejaculation	Placebo (437)	280 (64.1)	157 (35.9)
	Tadalafil 5 mg (124)	69 (55.7)	55 (44.4)
	Tadalafil 10 mg (237)	93 (39.2)	144 (60.8)§
	Tadalafil 20 mg (714)	201 (28.2)	513 (71.9)§
IIEF-Q10: orgasm	Placebo (507)	331 (65.3)	176 (34.7)
	Tadalafil 5 mg (136)	74 (54.4)	62 (45.6)¶
	Tadalafil 10 mg (288)	109 (37.9)	179 (62.2)§
	Tadalafil 20 mg (881)	267 (30.3)	614 (69.7)§

**IIEF-Q9: 'Over the past 4 weeks, when you had sexual stimulation or intercourse, how often did you ejaculate?'*
†IIEF-Q10: 'Over the past 4 weeks, when you had sexual stimulation or intercourse, how often did you have the feeling of orgasm or climax?' ‡Patients were considered to have improved if EjD or OD was no longer severe at 12 weeks. §$P < 0.001$; ¶$P = 0.020$ vs placebo by chi-squared tests.

Discussion

Ejaculatory and orgasmic dysfunction were common at baseline in more than 3000 patients enrolled in international clinical trials of tadalafil for ED. Treatment with tadalafil 10 or 20 mg as needed was associated with significant improvement in ejaculatory and orgasmic function (vs placebo) from baseline to 12 weeks, irrespective of baseline ED, EjD or OD severity. Subjects who continued to experience severe EjD or OD at study completion reported less marked improvements in sexual satisfaction than those whose ejaculatory and orgasmic function improved. For each degree of increasing EjD and OD severity (from minimal/no to moderate; and from moderate to severe) after 12 weeks of therapy with tadalafil 20 mg, there was about a 20% mean decrease in sexual satisfaction on the IIEF-IS and IIEF-OS domains and a ≈40% decline in sexual satisfaction on SEP5.

On the basis of our exploratory analysis, approximately two of three subjects with severe EjD or OD at baseline experienced improvements in ejaculatory and orgasmic function after tadalafil treatment, compared with about one of three in the placebo group. Similarly, ≈70% of per-patient responses to SEP5 were 'yes' among subjects who received tadalafil 20 mg treatment and had minimal or no EjD or OD at study completion, compared with about 30% on placebo. Our observed placebo effect suggests that there is a subjective dimension to self-reported improvements in ejaculatory and orgasmic function (as well as in erectile function) among patients with ED who participated in tadalafil clinical trials. However, it is unclear at this point if other factors, such as hypogonadism, contributed to the observed placebo effect.

Magnitudes of improvements in self-reported ejaculatory function (IIEF-Q9) and orgasmic function (IIEF-Q10) were numerically greater in subjects with more severe EjD or OD at baseline. The observed LS mean increases in IIEF-Q9 and IIEF-Q10 scores of 2.0 after tadalafil 20 mg treatment in subjects with severe EjD or OD at baseline were

Fig. 3 Least-squares mean changes from baseline to study endpoint (week 12 or LOCF) in per-patient percentage of 'yes' responses to SEP5 ('Were you satisfied overall with the sexual experience?') among subjects with different degrees of EjD at study completion. Operational definitions of EjD were as follows: severe EjD, a response of 1 ('almost never/never') or 2 ('a few times/much less than half the time') to IIEF-Q9 ('Over the past 4 weeks, when you had sexual stimulation or intercourse, how often did you ejaculate?'); moderate EjD, a response of 3 ('sometimes/about half the time') to IIEF-Q9; and minimal/no EjD, a response of 4 ('most times/much more than half the time') or 5 ('almost always/always') to IIEF-Q9. *$P < 0.05$ vs placebo. Numbers below the axis represent patients with available data.

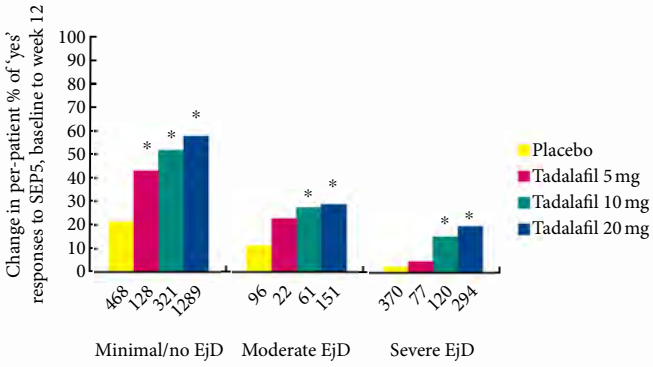
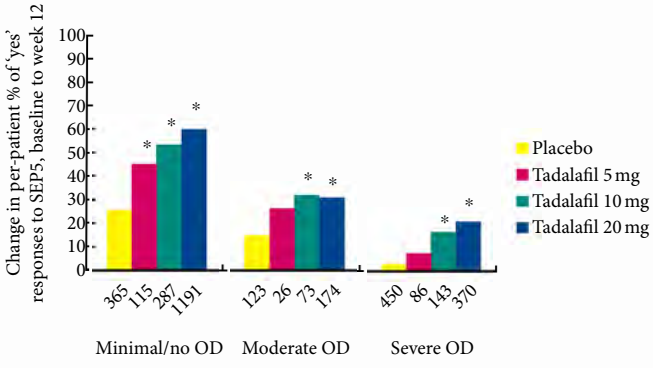


Fig. 4 Least-squares mean changes from baseline to study endpoint (week 12 or LOCF) in per-patient percentage of 'yes' responses to SEP5 ('Were you satisfied overall with the sexual experience?') among subjects with different degrees of OD at study completion. *$P < 0.05$ vs placebo. Operational definitions of OD were as follows: severe OD, a response of 1 ('almost never/never') or 2 ('a few times/much less than half the time') to IIEF-Q10 ('Over the past 4 weeks, when you had sexual stimulation or intercourse, how often did you have the feeling of orgasm or climax?'); moderate OD, a response of 3 to IIEF-Q10 ('sometimes/about half the time'); and minimal/no OD, a response of 4 ('most times/much more than half the time') or 5 ('almost always/always') to IIEF-Q10. *$P < 0.05$ vs placebo. Numbers below the axis represent patients with available data.



consistent with a potentially clinically meaningful categorical improvement in the average patient, from a score of 1 ('almost never/never') to 3 ('sometimes/about half the time'), with regard to ejaculation or orgasm after sexual stimulation.

Patients with moderate dysfunction experienced significant, but smaller and not dose-related, improvements with tadalafil (vs placebo). On the other hand, patients with minimal or no baseline EjD or OD, who had the least severe symptoms and hence the least room for improvement (i.e. 'ceiling effect'), experienced worsening ejaculatory and orgasmic function with placebo, which was significantly blunted by tadalafil treatment.

One limitation of the present study is our use of an instrument with unknown performance to measure responses to treatment for EjD and OD. There is a general paucity of office-based patient self-reported and other subjective measures of male sexual function apart from erectile function. The Male Sexual Health Questionnaire has specific questions about ejaculatory and orgasmic function; however, it has not been fully validated. Clearly, there is a need for validated instruments to measure ejaculatory and orgasmic function.

The promising, but preliminary and hypothesis-generating, findings of the present study suggest that tadalafil could help to meet patient needs beyond the ability to improve erections, such as ejaculation, orgasm and sexual satisfaction, as well as helping to achieve reproductive aims, where ejaculatory difficulties are not secondary to irreversible organic factors. That residual EjD or OD after treatment for ED was associated with decreased sexual satisfaction suggests that improving (or restoring) ejaculatory or orgasmic function could constitute an important patient-related outcome beyond improving erectile function.

Potential mechanisms by which tadalafil might improve ejaculatory and orgasmic function need to accommodate the multifactorial ('psycho-neuro-endocrine' [4]) physiology of these variables and the complex aetiology of EjD and OD. At a biopsychosocial level, the use of the long-acting PDE-5 inhibitor tadalafil might permit a relaxed encounter and a prolonged period of intimacy (and lengthier sexual encounter), promoting ejaculation and orgasm by increasing the capacity for, and amount of, sexual stimulation and also heightening the male patient's sensitivity to internal cues to reach ejaculation and orgasm. This mechanism would be consistent with Rowland and co-workers' concept of inhibited ejaculation resulting from an uncoupling of a reduced subjective and largely maintained genital reaction in sexual arousal [28]. In addition, patients with EjD (mainly PE) and OD who have received PDE-5 inhibitors have reported a greater sense of

Table 5 Least-squares mean changes from baseline to study endpoint (week 12 or LOCF) in IIEF satisfaction variables by treatment group and endpoint EjD or OD.

Subgroup/variable	As-needed treatment group			
	Placebo	Tadalafil 5 mg	Tadalafil 10 mg	Tadalafil 20 mg
Endpoint EjD (IIEF response)				
IIEF-IS*				
Severe (1 or 2)	0.6	1.1	1.6†	1.8†
Moderate (3)	1.4	2.4	2.4†	2.6†
Minimal or no (4 or 5)	2.5	4.0†	4.5†	4.8†
IIEF-OS*				
Severe (1 or 2)	−0.3	0	0.6†	0.9†
Moderate (3)	0.7	1.2	1.6†	1.6†
Minimal or no (4 or 5)	1.7	3.1†	3.1†	3.4†
Endpoint OD (IIEF response)				
IIEF-IS				
Severe (1 or 2)	0.5	1.2†	1.7†	1.9†
Moderate (3)	2.1	2.4	2.7	2.8†
Minimal or no (4 or 5)	2.8	4.3†	4.5†	4.9†
IIEF-OS				
Severe (1 or 2)	−0.2	0.6†	0.7†	1.1†
Moderate (3)	1.2	1.4	1.9†	1.9†
Minimal or no (4 or 5)	1.9	3.1†	3.2†	3.5†

**IIEF-IS is the mean of responses to IIEF-Q6–8; scores range from 0 [worst] to 15 [best]; IIEF-OS is the mean of responses to IIEF-Q13–14; scores range from 2 ['very dissatisfied'] to 10 ['very satisfied']. †P < 0.05 vs placebo.*

control over their erections as well as their ejaculatory and orgasmic functions [18,29,30]. At a physiological level, the key target of PDE-5 inhibitors' activities – the cyclic guanosine monophosphate (cGMP) second messenger of nitric oxide – plays a role in contractility of the male genital tract and could modulate central processing of neural inputs that facilitate orgasm.

It has not been shown that tadalafil crosses the blood–brain barrier; however, oral administration of tadalafil in animals has effects on the central nervous system [31]. Thus, it is possible that the observed effects of tadalafil on EjD and OD result from tadalafil's action on neuromuscular regulation of ejaculatory and orgasmic function.

Further studies of patients with EjD or OD (who may or may not have ED) are necessary to conclude reliably that tadalafil exerts favourable effects on ejaculatory and orgasmic function that transcend its effects on erectile function. The concept (or 'construct') of sexual satisfaction is highly subjective and, at the time of writing, has not been conclusively linked to more objective, observable outcome variables. Although all 17 studies analysed herein involved as-needed treatment with tadalafil (or placebo), trials assessing once-daily tadalafil generated similar findings (data not shown).

A limitation of the present analyses is that they might have underestimated the degrees of ejaculatory and orgasmic dysfunction compared with men who do not seek medical attention for EjD until after they have experienced ED. Furthermore, some potentially beneficial effects of tadalafil could have been indirect, and the present analysis could not control for all covariates. For instance, the effects of tadalafil on orgasmic function could have been partly secondary to the agent's effects on ejaculatory function because attention to, and cerebral processing of, pleasurable contractions of the male genital tract during ejaculation contribute to the development of orgasm; the intensity of orgasm could also be potentiated by the robustness of the emission and ejaculatory processes. Our study focused on tadalafil; however, a class effect of PDE-5 inhibitors cannot be ruled out. Further research is needed to clarify the physiological mechanisms of male ejaculation and orgasm in general, and the potential effects of PDE-5 inhibitors on these mechanisms in particular.

In conclusion, difficulties with ejaculation and orgasm were associated with reduced sexual satisfaction in this exploratory, retrospective, pooled-data analysis of patients enrolled in double-blind clinical trials of tadalafil for treating ED. Treatment with tadalafil was associated with significant increases in ejaculatory and orgasmic function (vs placebo), irrespective of baseline ED severity, and also with significantly increased sexual satisfaction. Residual (post-treatment) EjD or OD was associated with a decrease in patients' sexual satisfaction. These findings warrant corroboration in further prospective, placebo-controlled clinical trials involving patients presenting with ejaculatory or orgasmic dysfunction (with or without ED).

Fig. 5 Mean baseline and endpoint (or LOCF) responses to measures of patient satisfaction according to endpoint ejaculatory function among subjects receiving tadalafil 20 mg as needed before sexual activity in placebo-controlled trials of ED. Measures of patient satisfaction included the IIEF-IS domain (**A**); the IIEF-OS domain (**B**); and SEP5, 'Were you satisfied overall with the sexual experience?' (**C**). Operational definitions of EjD were as follows: severe EjD, a response of 1 ('almost never/never') or 2 ('a few times/much less than half the time') to IIEF-Q9 ('Over the past 4 weeks, when you had sexual stimulation or intercourse, how often did you ejaculate?'); moderate EjD, a response of 3 to IIEF-Q9 ('sometimes/about half the time'); and minimal/no EjD, a response of 4 ('most times/much more than half the time') or 5 ('almost always/always') to IIEF-Q9.

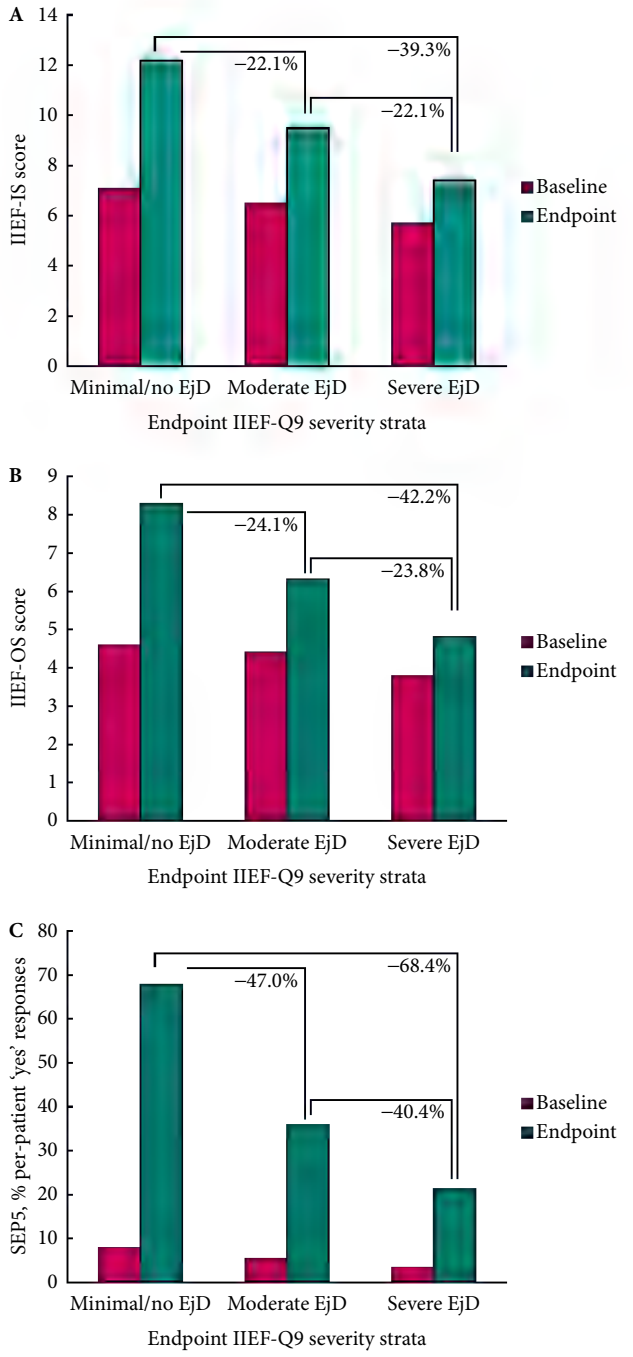
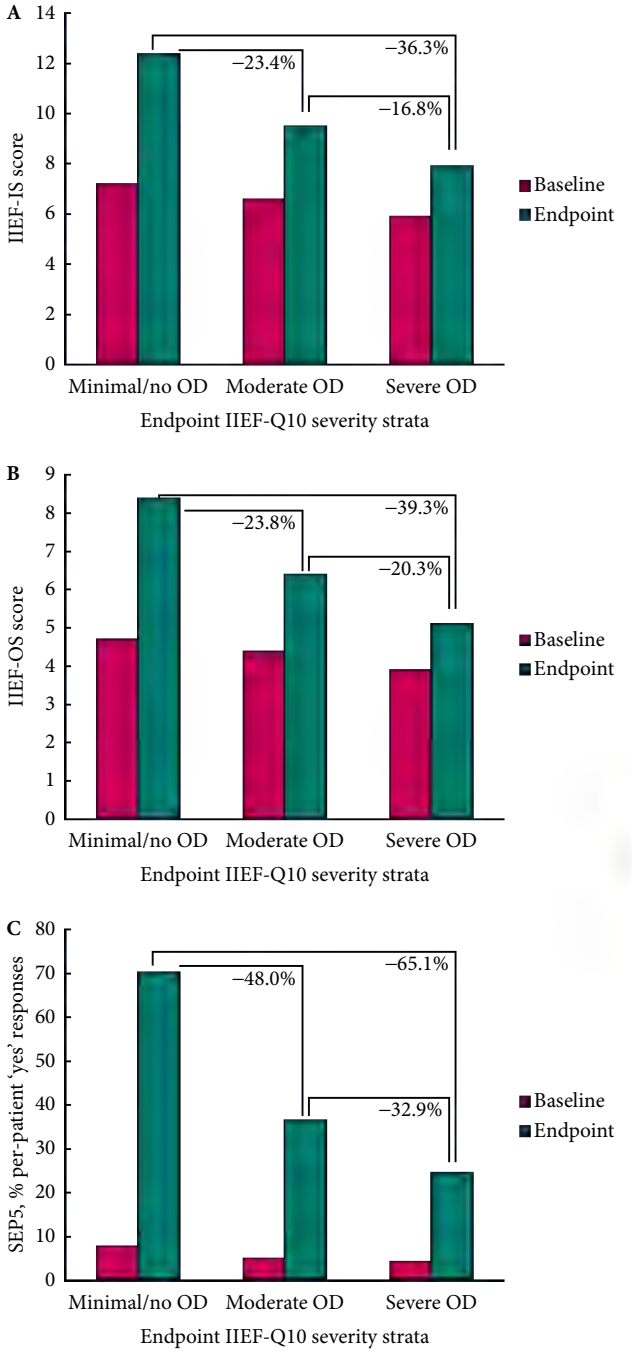


Fig. 6 Mean baseline and endpoint (or LOCF) responses to measures of patient satisfaction according to endpoint orgasmic function among subjects receiving tadalafil 20 mg as needed before sexual activity in placebo-controlled trials of ED. Measures of patient satisfaction included the IIEF-IS domain (**A**); the IIEF-OS domain (**B**); and the SEP5, 'Were you satisfied overall with the sexual experience?' (**C**). Operational definitions of OD were as follows: severe OD, a response of 1 ('almost never/never') or 2 ('a few times/much less than half the time') to IIEF-Q10 ('Over the past 4 weeks, when you had sexual stimulation or intercourse, how often did you have the feeling of orgasm or climax?'); moderate OD, a response of 3 ('sometimes/about half the time') to IIEF-Q10; and minimal/no OD, a response of 4 ('most times/much more than half the time') or 5 ('almost always/always') to IIEF-Q10.



Acknowledgements

Assistance with manuscript preparation was provided by Stephen W. Gutkin, Rete Biomedical Communications Corp. (Wyckoff, NJ, USA), with support from Eli Lilly.

Conflict of Interest

The base studies, as well as the present analysis and report, were supported by Eli Lilly and Company (Indianapolis, IN, USA). DAP and AB are paid investigators and/or consultants/advisors/speakers for the study sponsor. PKP and SDW are employees of, and minor shareholders in, Eli Lilly.

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Abbreviations: DE, delayed ejaculation; EjD, ejaculatory dysfunction; ED, erectile dysfunction; IIEF, International Index of Erectile Function; IIEF-IS and IIEF-OS, IIEF intercourse satisfaction and overall satisfaction domains; LOCF, last observation carried forward; LS, least-squares; OD, orgasmic dysfunction; PDE-5, phosphodiesterase type-5; PE, premature ejaculation; SEP, Sexual Encounter Profile.

Concordance among sperm deoxyribonucleic acid integrity assays and semen parameters

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Objective: To assess the concordance of sperm chromatin structure assay (SCSA) results, epifluorescence TUNEL assay results, and standard semen parameters.
Design: Prospective, observational study.
Setting: Tertiary referral andrology clinic.
Patient(s): A total of 212 men evaluated for subfertility by a single physician.
Intervention(s): Clinical history, physical examination, semen analysis, SCSA, and TUNEL assay.
Main Outcome Measure(s): Spearman's rank correlation coefficients (r) between SCSA DNA fragmentation index (DFI), percentage TUNEL-positive sperm, and semen analysis parameters.
Result(s): There was a positive correlation between SCSA DFI and TUNEL ($r = 0.31$), but the strength of this correlation was weaker than has previously been reported. The discordance rate between SCSA and TUNEL in classifying patients as normal or abnormal was 86 of 212 (40.6%). The SCSA DFI was moderately negatively correlated with sperm concentration and motility. The TUNEL results were unrelated to standard semen parameters.
Conclusion(s): The SCSA DFI and percentage TUNEL-positive sperm are moderately correlated measures of sperm DNA integrity but yield different results in a large percentage of patients. The DFI is well-correlated with semen analysis parameters, whereas TUNEL is not. These data indicate that the SCSA and TUNEL assay measure different aspects of sperm DNA integrity and should not be used interchangeably. (Fertil Steril® 2015;104:56–61. ©2015 by American Society for Reproductive Medicine.)
Key Words: DNA fragmentation, in situ nick-end labeling, infertility (male), semen analysis, spermatozoa

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Diagnosis and classification of male subfertility depends in large part on quantitative assessment of semen quality. Standard semen analysis (SA) performed according to protocols published by the World Health Organization (WHO) (1) is by far the most commonly utilized such test. However, SA has several significant limitations, including poor prognostic performance in predicting outcomes of natural and assisted reproductive cycles (2) and high levels of intraindividual variability (1). The limited clinical value of standard SA underscores the need for tests that enhance the ability to diagnose male factor infertility.

The critical importance of sperm DNA integrity for human fertility has been increasingly recognized over the past 15 years (3); and tests for the detection of sperm DNA damage have emerged as additional measures of semen quality. Sperm DNA damage is more prevalent among subfertile couples (4), and higher levels of sperm DNA damage are associated with impaired spermatogenesis (5). A growing body of literature has linked results of sperm DNA integrity assays with rates of natural conception (6), conception after IUI (7), pregnancy loss after assisted reproductive cycles (8), and rates of conception after varicocele repair (9).

The most commonly used of several available tests of sperm DNA integrity are the sperm chromatin structure assay (SCSA) and the TUNEL

Received February 11, 2015; revised March 14, 2015; accepted April 15, 2015; published online May 16, 2015.
P.J.S. has nothing to disclose. C.C. has nothing to disclose. A.M. has nothing to disclose. A.B. has nothing to disclose. D.A.P. has nothing to disclose. M.G. has nothing to disclose.
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Fertility and Sterility® Vol. 104, No. 1, July 2015 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2015.04.023

assay. The SCSA uses flow cytometry to measure the stability of double-stranded sperm chromatin when exposed to a denaturant (10). Test results are given as the percentage of sperm with denatured (single-stranded) DNA after denaturant exposure, which is termed the DNA fragmentation index (DFI). In the TUNEL assay, individual sperm with native DNA strand breaks are stained or labeled with a fluorochrome and detected by either fluorescent microscopy or flow cytometry (11). Results are given as the percentage of TUNEL-positive (or negative) sperm.

Though often used and discussed interchangeably as measures of sperm DNA damage, the SCSA and TUNEL assay measure different characteristics of sperm DNA. Furthermore, even flow cytometry and epiflourescence-based TUNEL assays may be measuring different aspects of sperm DNA damage. Flow cytometry does not discriminate sperm according to morphology, and the DFI reported by such assays indicates the percentage of all sperm with native DNA strand breaks, regardless of sperm morphology. In comparison, TUNEL assays using epifluorescence microscopy combined with contrast-phase or Nomarski optics, such as the assay used in this study, allow for direct visualization of sperm morphology and enable reporting of the percentage of morphologically normal sperm with native DNA strand breaks.

Previously published studies describing the concordance of the SCSA and TUNEL assay with each other and with standard semen parameters have been limited by low numbers of patients and inconsistent results (12–15). The present study is the largest to date evaluating the relationships between SCSA DFI, percentage TUNEL-positive sperm, and standard semen parameters.

MATERIALS AND METHODS

Patient Selection and Evaluation

This research protocol was approved by the institutional review board at Weill Cornell Medical College of Cornell University. This was a prospective analysis of baseline semen quality for 212 subfertile men evaluated by a single physician from 2009 to 2014. Starting in 2009, sperm DNA integrity testing with both the TUNEL assay and SCSA was offered to all men consecutively evaluated for subfertility. Both tests were routinely ordered as part of the study design, but performance of testing was subject to patient compliance with the physician recommendation for testing. Only patients who underwent standard SA and sperm DNA integrity testing with both the SCSA and TUNEL assay were included.

The baseline clinical evaluation for each patient included a comprehensive history and complete physical examination performed in a warm room after placing a heating pad on the scrotum to relax the dartos muscle. Testicular volumes were measured with an orchidometer. Serum FSH and total early morning T levels were assessed by a peripheral venous serum sample taken between 8:00 AM and 10:30 AM. Semen analysis was performed manually using the 1999 WHO protocol. Semen was collected in a specially designated room in our embryology laboratory, with the aid of audiovisual stimulation.

Sperm DNA Integrity Testing

The SCSA was performed by the proprietary SCSA diagnostics laboratory according to the original method described by Evenson et al (10). Patients used a prepackaged kit to collect, freeze, and mail semen samples produced at home to the SCSA diagnostics laboratory. Semen samples for the SCSA were collected within a range of 1–6 weeks from the time of semen collection for standard SA and TUNEL analysis. Frozen samples were thawed, diluted, exposed to acid detergent, and then stained with acridine orange. The fluorescence patterns of 5,000 sperm cells were sorted using flow cytometry and analyzed using proprietary software to determine the DFI of each sample. Values for SCSA DFI $\geq$25% were considered abnormal.

The TUNEL assay was performed as previously described (16), and TUNEL was performed on the same semen sample provided for standard SA. Four smears from each semen sample were prepared on glass slides and air-dried. The In Situ Cell Death Detection Kit with Fluorescein isothiocyanate (FITC; Roche Diagnostics) was used with modifications. Each slide was fixed with 4% paraformaldehyde (1 mL) in phosphate-buffered saline (PBS) solution and incubated at room temperature for 1 hour. Slides were washed with ice-cold PBS, then permeabilized with Triton X in 0.1% sodium citrate for 5 minutes. Slides were again washed with PBS, then incubated with a mixture of the TUNEL enzyme solution containing terminal deoxynucleotidyl transferase plus TUNEL labeling solution containing deoxyuridine triphosphate. A Parafilm M strip (Alcan Packaging) was applied to each slide, and the slides were incubated in a dark, moist chamber at 37°C for 1 hour. After labeling, slides were taken out of the chamber, the Parafilm M was removed, and the cells were washed with PBS. Vectashield (Vector Laboratories) with 4′,6-diamidino-2-phenylindole (DAPI) was applied to each slide for DNA counterstaining, and a cover slip was applied. Cells were allowed to stain overnight. Two negative and two positive controls were tested with each batch.

Slides were analyzed using an epifluorescent microscope at 400× magnification. The number of DAPI-positive cells was counted, then, in the same field, the number of FITC-positive cells was recorded. At least 100 DAPI-positive cells were counted for a single tally. The number of FITC-positive cells detected was divided by DAPI-positive cells × 100 to produce the percentage of TUNEL-positive cells (containing fragmented DNA), and at least four separate fields were analyzed. Only sperm with presence of normal midpiece, tail, and normal-appearing head were counted for TUNEL assay because such sperm would be normally chosen during IVF. In this respect the TUNEL assay performed in our laboratory uses “strict” criteria (17). The TUNEL tests were considered abnormal when the percentage of TUNEL-positive sperm was $\geq$7%.

Statistical Analysis

Correlations between SCSA DFI, percentage TUNEL-positive sperm, and WHO semen parameters were analyzed by nonparametric Spearman’s rank correlation coefficients (r) using GraphPad Prism 5 software. The discordance rate

TABLE 1

Spearman's correlation coefficients (*r*) between SCSA DFI, percentage TUNEL-positive sperm, and WHO semen parameters.

Variable	Semen volume	Sperm concentration	% Motility	% Normal morphology (WHO 1999)
SCSA DFI	0.06 (*P*=.4)	0.34 (*P*< .000001)	0.45 (*P*< .000001)	0.1 (*P*=.19)
% TUNEL-positive sperm	0.1 (*P*=.15)	0.01 (*P*=.85)	0.15 (*P*=.03)	0.06 (*P*=.43)

Stahl. Concordance of sperm DNA assays. Fertil Steril 2015.

between the SCSA and TUNEL assay in classifying sperm DNA integrity as normal or abnormal was determined using laboratory-recommended cutoff values for each assay.

RESULTS

The mean age of the patient cohort was 39.3 years (SD 6.2, range 25–58 years). Unilateral or bilateral varicoceles were present in 166 of 212 patients, with the size of the largest varicocele being grade 1 in 48 men, grade 2 in 87, and grade 3 in 31. No patients had been exposed to prior chemotherapy or radiation, and none had current or prior testicular malignancies.

Mean semen volume was 2.7 mL (SD 1.3, range 0.2–8.1 mL). Mean sperm concentration was 46.5 $\times 10^6$/mL (SD 38.2, range 0.01–184 $\times$ 10^6/mL). Mean overall motility was 51.2% (SD 16.7%, range 5%–91%). Mean percentage normal morphology according to WHO 1999 criteria was 9.4% (SD 9.3%, range 0–52%). Mean serum FSH was 6.1 IU/L (SD 4.8, range 0.1–29.9 IU/L). The mean SCSA DFI was 20.1% (SD 15.1%, range 2%–81%). The mean percentage TUNEL-positive sperm was 12.1% (SD 8.0%, range 2%–50.8%).

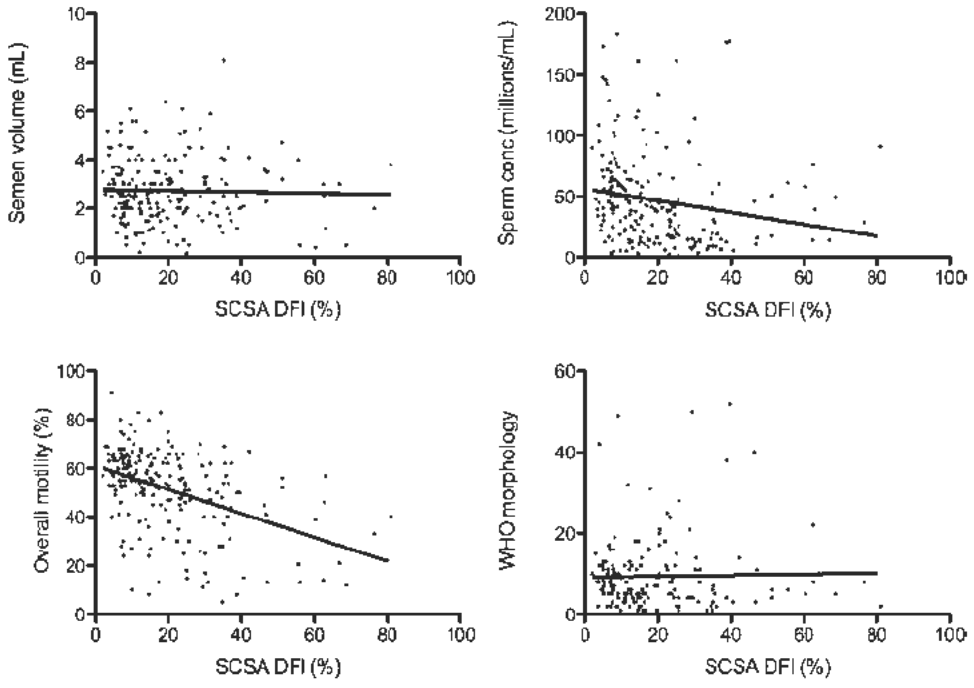
Spearman's correlation coefficients indicating correlations between SCSA DFI and percentage of TUNEL-positive sperm with WHO SA parameters are given in Table 1 and are visually displayed in Figures 1 and 2. The SCSA DFI exhibited moderate and strong negative relationships with sperm concentration and motility, respectively. Correlations between TUNEL assay results and WHO semen parameters were not observed or were negligible.

There was a moderate positive correlation between SCSA DFI and the percentage TUNEL-positive sperm (r = 0.314, P< .00001), but this correlation was weaker than has previously been reported. The discordance rate between SCSA DFI and the percentage of TUNEL-positive sperm in classifying patients as normal or abnormal was 86 of 212 (40.6%) (Fig. 3).

DISCUSSION

Assays evaluating native sperm DNA strand breaks or the susceptibility of sperm DNA to denaturation have been developed as measures of sperm DNA integrity. The SCSA and TUNEL assay are the most commonly utilized such tests. Other

FIGURE 1



Relationships between SCSA DFI and standard semen parameters. Solid lines represent the best-f t linear regression lines.

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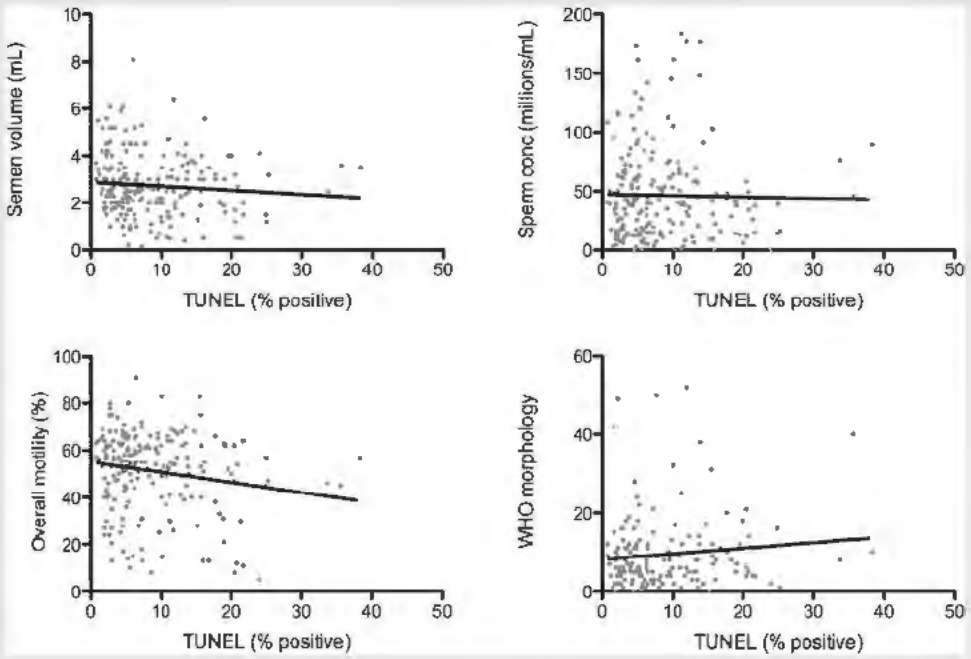
FIGURE 2



Relationships between the percentage of TUNEL-positive sperm and standard semen parameters. Solid lines represent the best-fit linear regression lines.

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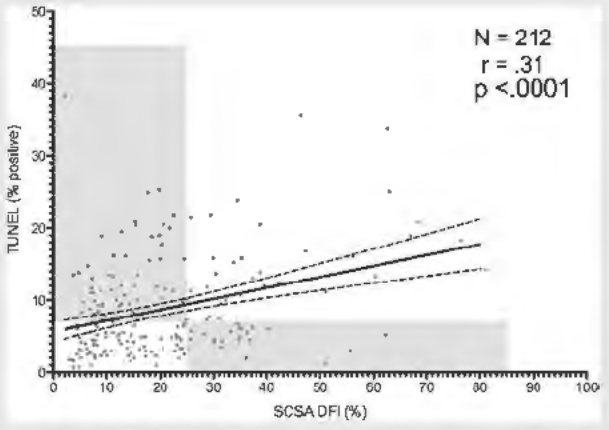
well-described assays include the single-cell gel electrophoresis assay (COMET) (18) and the sperm chromatin dispersion test (19). These four tests and others have been espoused as alternative assessments of semen quality that are less variable than standard semen parameters and can detect occult male factors not readily identifiable with conventional semen testing (20). Utilization of sperm DNA integrity testing in clinical practice has become common, though at present the currently available evidence is insufficient to support routine clinical use (21).

FIGURE 3



Concordance between SCSA DFI and TUNEL assay results. Grey shaded areas indicate patients who would be labeled as normal by one assay and abnormal by the other. Solid and dashed lines represent the best-fit linear regression line and its 95% confidence interval, respectively. Grey shaded boxes indicate patients with abnormal results from one assay but normal results from the other.

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The enthusiasm for integration of sperm DNA integrity assays into clinical practice is based on studies that have demonstrated associations between sperm DNA integrity and reproductive outcomes. A recent systematic review of sperm DNA integrity testing concluded that such tests predict pregnancy after natural conception, IUI, and IVF but do not impact pregnancy rates after IVF/intracytoplasmic sperm injection (22). However, a recently presented study from our institution revealed no live births after IVF/intracytoplasmic sperm injection when sperm TUNEL testing exceeded 25% (17). These data suggest that testing may be useful in counseling of subfertile couples about the likelihood of achieving pregnancy and in selection of assisted reproductive techniques.

Selection of a particular sperm DNA integrity assay for use in the clinical evaluation of a subfertile couple is driven by clinician preference and institutional assay availability. Guidelines for assay selection do not exist. Patients who undergo sperm DNA integrity testing are classified according to reference values provided by the laboratory performing each test as having either normal or abnormal sperm DNA integrity, and clinical decisions are made based on these results in the context of the existing literature. Although the available assays are methodologically distinct, they are often used and discussed interchangeably as nonspecific measures of sperm DNA integrity.

The notion that sperm DNA integrity assays may be used interchangeably in clinical decision making is based on several studies that have examined interassay concordance. These prior studies have consistently demonstrated strong correlations between SCSA DFI and TUNEL results, with correlation coefficients ranging from 0.56 to 0.99 (Supplemental Table 1, available online) (12, 13, 15, 23, 24). However, most of these studies are limited by small numbers of patients (range 25–60 patients). In the present study, which includes more patients than all of the previously reported studies combined, the concordance between SCSA and TUNEL results was far weaker ($r = 0.31$) than has previously been reported. Moreover, there was high likelihood of a patient being categorized as having normal sperm DNA by one assay and damaged sperm DNA by the other (41%).

One explanation for the observed discordance between SCSA and TUNEL assay results is the significant conceptual difference between these two tests. The SCSA is a test of sperm DNA stability upon exposure to a denaturant, whereas the TUNEL assay detects in situ pre-existing DNA strand breaks under neutral conditions. It has been said that the SCSA is therefore a measure of "potential" sperm DNA damage, and that in contrast the TUNEL assay detects "real" sperm DNA damage (15). Furthermore, the addition of contrast microscopy to exclude morphologically abnormal sperm from DFI calculations may also have altered the performance characteristics of the TUNEL assay and its correlation with the SCSA, which does not account for sperm morphology at all.

Another explanation, which may also contribute to the differences between our results and prior studies that have shown stronger correlations between SCSA and TUNEL, is that neither the SCSA nor the TUNEL assay have been methodologically standardized to provide optimal clinical information and predictive value. Results on SCSA have been shown to vary according to several protocol and laboratory factors, including use of ice for postthaw semen sample incubation and even by the laboratory technician performing the assay (25). Furthermore, several different protocols are available and used for labeling DNA strand breaks during the TUNEL assay, and detection of DNA-damaged sperm may be performed using flow cytometry or fluorescent microscopy.

The choice of sperm DNA integrity tests should depend on local familiarity with the protocols and the clinical scenario. In academic centers like ours, which deal with high numbers of severely oligospermic men, flow cytometry–based assays are not feasible because they require 10,000 to 100,000 sperm per run. In contrast, the TUNEL assay can be performed on samples containing fewer than 1,000 sperm after centrifugation. In addition, the epiflourescence-based TUNEL assay is the only assay that allows for use of the same methodology for analysis of ejaculated and surgically retrieved sperm, which may prove to be useful in selection of sperm to use for assisted reproduction.

Although our study is the largest study of sperm DNA integrity assay concordance published to date, there are several methodologic limitations that may have influenced our results. As a tertiary referral center for patients with varicocele, our study population was composed of a higher percentage of patients with varicoceles (78%) than would be expected in the general population of subfertile men. Varicoceles may exert discriminate adverse effects on TUNEL and SCSA results, and it is possible that a less-biased patient population would have resulted in a stronger observed concordance between SCSA and TUNEL results. Another important methodologic limitation of our study is that the SCSA and TUNEL assay were performed on different semen samples. Intraindividual variation in semen quality may have influenced the observed concordance between TUNEL and SCSA results.

In conclusion, the observed concordance between the SCSA DFI and the percentage of TUNEL-positive sperm was weaker in this study than has been previously described. These assays are moderately correlated measures of sperm DNA integrity but yield conflicting results in a large percentage of patients. The SCSA DFI is well-correlated with SA parameters, whereas TUNEL is not. These data indicate that the SCSA and TUNEL assays measure different aspects of sperm DNA integrity and should not be used or discussed interchangeably. At this time there are insufficient available data to guide selection of specific sperm DNA integrity assays for use in clinical practice. Prospective studies investigating the correlations of specific sperm DNA assay results with clinical and reproductive outcomes are needed to help clinicians select specific assays that will guide patient counseling or change clinical care.

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SUPPLEMENTAL TABLE 1

Previously reported studies evaluating the concordance of SCSA DFI and TUNEL.

Study (reference)[a]	No. of patients	Correlation (*r*)	*P* value
Gorczyca 1993 (13)	25	0.87	< .05
Sailer 1995 (23)	25	0.56	.004
Zini 2001 (12)	40	0.71	< .0001
Chohan 2006 (24)	60	0.90	< .001
Henkel 2010 (15)	52	0.99	< .0001

[a] Full reference citations can be found in main text.

Stahl. Concordance of sperm DNA assays. Fertil Steril 2015.

Current Management of Adolescent Varicocele

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The finding of varicocele in an adolescent male is common. Varicocele rarely causes symptoms and is often diagnosed on the routine physical examination. There is clear association between varicocele and male factor infertility; however, there is debate about whether, when, and whom to treat when present in adult or adolescent males. This review of the epidemiology, etiology, pathophysiology, and treatment of the adolescent with varicocele will provide the reader with tools to make appropriate decisions in dealing with this condition. [Rev Urol. *2001;3(3):120–133*]

Key words: Varicocele • Varicocelectomy • Testicular growth arrest • Male infertility • Retroperitoneal ligation • Microsurgical inguinal repair

A varicocele can be defined as an abnormal tortuosity and dilation of the veins of the pampiniform plexus (PP). Idiopathic varicocele is usually asymptomatic. It is noticed as an asymmetry in scrotal size, and presents as heaviness in the scrotum, or, rarely, with testicular pain. In most cases, the adolescent is unaware of the varicocele, and it is discovered during a regular physical examination or examination for military service.[1-4]

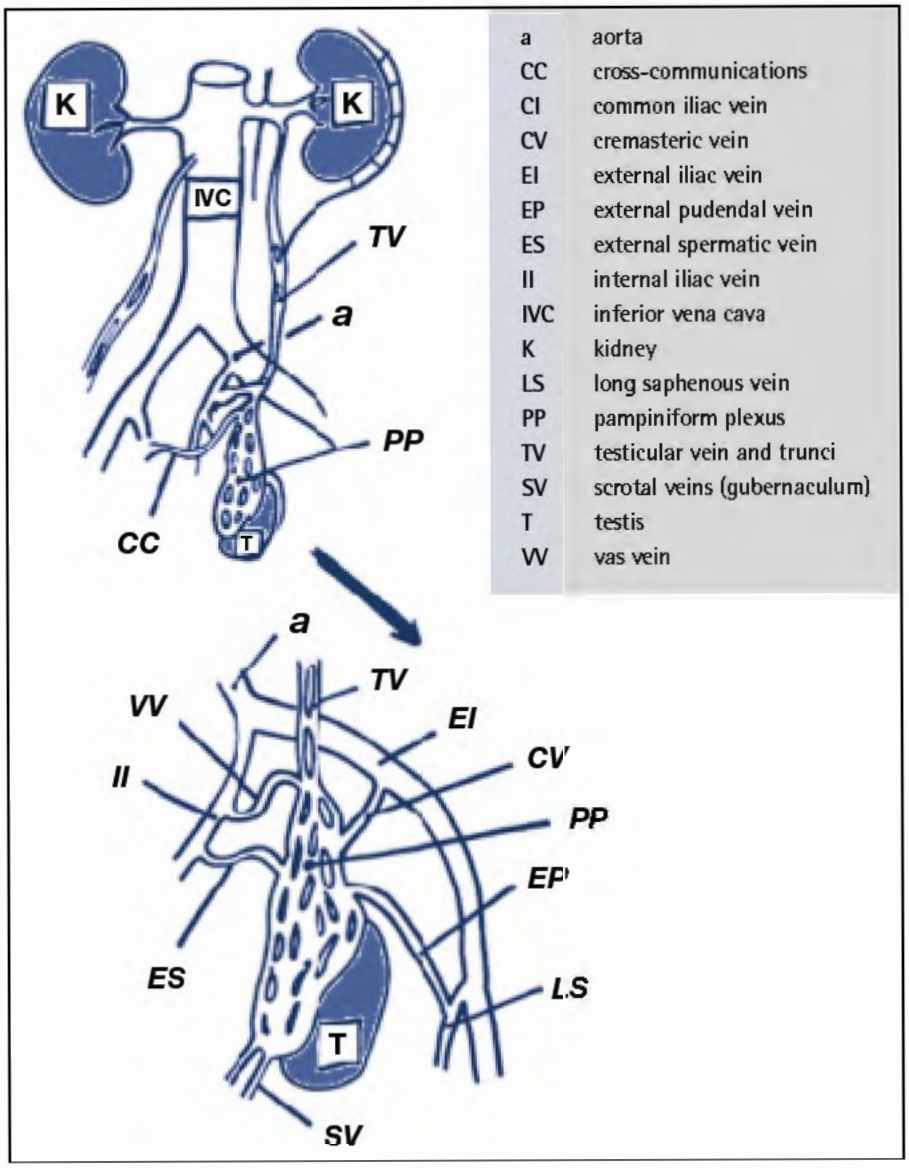
The incidence of high-grade varicocele is approximately 5% throughout the world.[5] Varicocele is associated with a time-dependent growth arrest in adolescents and adult males.[6] There is a clear association between varicocele, infertility, and testicular growth arrest.[7-9] It is also known that varicocelectomy can reverse growth arrest in adolescents.[10-13] This knowledge has raised the question of how best to manage adolescents with varicocele.

Adolescents do not present with infertility, and thus should prophylactic repair be performed to prevent infertility in the future? Who would benefit the most from varicocelectomy: adolescents with testicular growth arrest or any adolescent with varicocele? Is it better to wait for a semen analysis or offer earlier treatment based on testicular growth arrest? These questions can be only answered when we have a better understanding of the pathophysiology of varicocele.

Table 1
Incidence of Varicocele in General Population of Healthy Adolescents

Reference	No. of Patients	Age (y)	Incidence (Total)
Oster 1971[186]	837	10–19	16.2%
Steeno 1976[116]	4067	12–25	14.7%
Yerokhin 1979[187]	10,000	10–17	12.4%
Belloli 1993[9]	9861	10–16	16.0%
Niedzielski 1997[5]	2478	10–20	17.8%

Figure 1. *Anatomy of venous drainage from left testis. For abbreviations, see accompanying list.*



a	aorta
CC	cross-communications
CI	common iliac vein
CV	cremasteric vein
EI	external iliac vein
EP	external pudendal vein
ES	external spermatic vein
II	internal iliac vein
IVC	inferior vena cava
K	kidney
LS	long saphenous vein
PP	pampiniform plexus
TV	testicular vein and trunci
SV	scrotal veins (gubernaculum)
T	testis
VV	vas vein

This review presents the current literature on adolescent varicocele and provides guidelines to the clinician on how to manage adolescents with varicocele.

Epidemiology

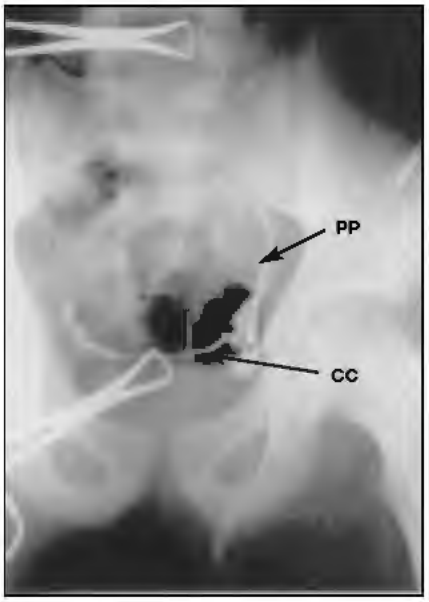
In the general population of healthy males, the overall incidence of varicocele (all grades) is 10% to 15%.[4,5,14,15] Approximately 30% to 50% of males with primary infertility have varicocele.[16–19] Varicocele is most common on the left side. It appears at early puberty, but can occasionally be found in preadolescent boys.[2,20] The incidence in older adolescents varies from 12.4% to 17.8%, with an average of 14.7% (Table 1), similar to the incidence in adult males.

Anatomy

The arterial blood supply to the testicle comes from the testicular artery, vasal artery, and cremasteric artery. At the level of the testis, all three arteries anastomose to allow adequate blood supply, even with the division of the testicular artery.[21,22]

Venous drainage (Figure 1) is more complicated, with many individual variations. Above the testis is a network of communicated veins called the pampiniform plexus, which drains via the testicular vein trunci, pudendal veins, and cremasteric veins.[23,24] In most cases, the testicular vein trunci form a single testicular vein entering the renal vein on the left and the inferior vena cava on the right. Venographic studies have demonstrated that the left testicular vein can rarely enter the inferior vena cava, and there are communications between the testicular vein and the inferior vena cava below the level of the renal veins.[25–27] There is also cross-communication between the left and right testicular venous systems (Figure 2).[26,28–30]

Figure 2. *Intraoperative venogram showing left to right cross-communicating veins. CC, cross-communications; PP, pampiniform plexus veins.*



Etiology

The predominance of left-side varicocele and the unique anatomy of the left testicular vein are the basis for several theories explaining the etiology of varicocele. The presence of venous valves was long believed to be a mechanism guarding against developing varicocele, and incompetence of the venous valve system was thought to be responsible for varicocele development. However, it was shown that there are males without varicocele who have an incompetent venous valve system and males with varicocele who have competent venous valves.[29]

Hydrostatic pressure difference could be a factor causing a left varicocele. Although the left testicular vein is longer than the right, the simple difference in hydrostatic pressure of a standing column of blood cannot be the only reason for development of varicocele, because all males would be affected.

The "nutcracker effect" is thought to occur when the testicular vein is compressed between the superior mesenteric artery and aorta. The increase in hydrostatic pressure results in varicocele formation. Although a high left renal vein to vena cava pressure gradient is noted in patients with varicocele, it is not a consistent feature.[31,32]

More recently, it has been hypothesized that increased arterial blood flow to the testis at puberty exceeds venous capacity, resulting in venous dilatation and varicocele.[33,34] The results of animal studies to support this interesting theory are conflicting, and confirmation in humans will be necessary.[35]

The finding of elevated nitric oxide, a potent vasodilator, in the PP of males with varicocele raises another potential cause for the etiology of varicocele in the adolescent.[36]

Pathophysiology

The pathophysiology of varicocele can be studied in animal models by partial ligation of the left renal vein.[37] Many features of the human condition, such as increased temperature of the affected testis, increased arterial blood flow, and histopathologic changes, can be replicated in animal models.[38]

The following theories attempt to explain the deleterious effect of the varicocele on testicular function:

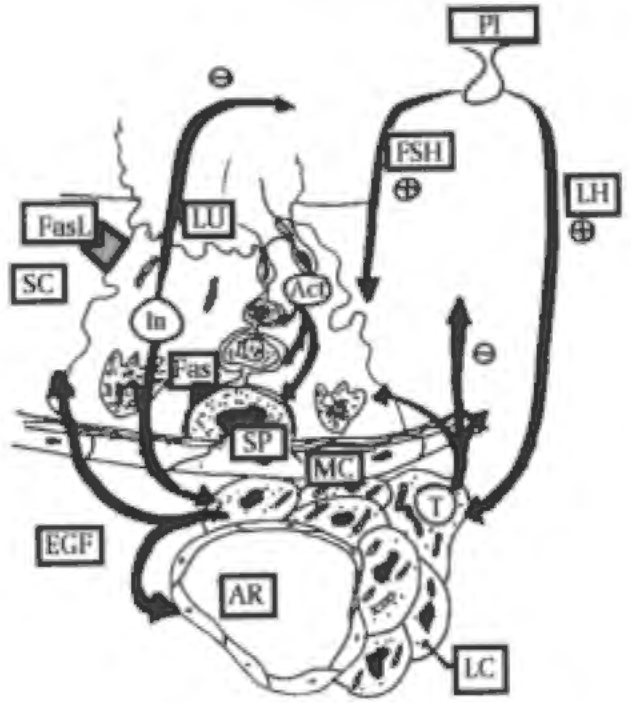
Hyperthermia. The presence of varicocele is associated with elevated scrotal and testicular temperature and altered spermatogenesis.[39] Experimental studies have shown that spermatogenesis occurs optimally at temperatures lower than body temperature. Many of the enzymes responsible for optimal DNA synthesis in the testis are temperature-dependent.[40,41] The scrotal position of the testis and the countercurrent cooling system provided by the PP surrounding the testicular artery allow for heat exchange and are responsible for regulating optimal temperature for spermatogenesis.[42] Stasis of blood in the varicocele with resultant increased temperature may be responsible for the deleterious effect of varicocele on spermatogenesis.[43] Increased temperature is associated with decreased number of spermatogonia (SP) and increased apoptosis of germinal epithelium cells.[44]

Abnormal blood flow. A current hypothesis assumes that increased blood flow through the testis can affect spermatogenesis.[45,46] An increase in hydrostatic pressure with a change in filtration pressure could considerably alter the composition of the interstitial fluid.[47] This conceivably could alter the intimate paracrine communications between the Leydig cells (LCs), peritubular myoid cells, and Sertoli cells (SCs), ultimately affecting spermatogenesis.[1] The myoid cells and capillary epithelium undergo pathological changes in association with varicocele that may effect transmembrane transport of substrates to the germinal epithelium.[48]

Hypoxia and "adrenal reflux." Stasis of blood in the PP could affect partial oxygen pressure and change aerobic metabolism in the testis. However, hypoxia has not been demonstrated in testicular venous blood sampling in humans or experimental animals.[49,50] Reflux of blood down the testicular vein has been demonstrated in patients with varicocele.[51-53] Therefore, exposure of the testis to adrenal or renal metabolites is hypothesized to be a cause for testicular damage. However, adrenal or renal metabolites at the level of the testis have not been documented.[37,54] Adrenalectomy in rats with experimental varicocele did not diminish the effects of the varicocele.[55,56] Thus the adrenal/renal reflux theory does not appear to be responsible for the testicular damage associated with varicocele.[55,57]

Figure 3. *Endocrine regulation of spermatogenesis. Sertoli cells under the influence of follicle-stimulating hormone regulate spermatogenesis by activin, androgen binding protein, and direct interactions with spermatogonia and spermatids. Leydig cells (LCs) regulate spermatogenesis by achieving a high intratesticular testosterone concentration. Epidermal growth factor (EGF) is produced by LCs and controls mitotic divisions of germinal epithelium. EGF also stimulates divisions of peritubular myoid cells that in return produce another paracrine hormone, peritubular myoid substance (PmodS). For abbreviations, see accompanying list.*

Act	activin	LU	lumen of seminiferous tubule
AR	arteriole	MC	myoid cells
EGF	epidermal growth factor	PI	pituitary gland, hypothalamus
FasL, Fas	ligand and receptor system involved in apoptosis of germ cells	SC	Sertoli cells
		SP	spermatogonia
FSH	follicle stimulating hormone	T	testosterone
In	inhibin	Circled minus sign	decrease (negative feedback)
LC	Leydig cell	Circled plus sign	increase (positive feedback)
LH	luteinizing hormone		



Endocrine imbalance. Puberty, spermatogenesis, and testicular development are regulated by the hypothalamic-pituitary-testicular axis. There is a wide array of endocrine abnormalities associated with varicocele. LCs are under the control of luteinizing hormone (LH) and are responsible for testosterone (T) production. Some studies have shown that the serum T level may be affected by varicocele; however, it is intratesticular testosterone that is important in the regulation of spermatogenesis.[58,59] In experimental animal models, varicocele can result in a decreased intratesticular testosterone level.[60] The results of human studies are mixed. Ando and colleagues found reduced serum T level in males with varicocele and increased serum T level after repair of the varicocele.[61–65] Swerdloff and Walsh, however, showed that there was no difference in testosterone level between males with and without a varicocele.[66]

Increased LH serum levels and an abnormal response to gonadotropin-releasing hormone (GnRH) could implicate a compromise of the hypothalamic-pituitary-gonadal axis involved in the control of T and spermatogenesis, a pattern similar to hypergonadotropic hypogonadism.[67,68] Increased LH level results in LC hyperplasia, a known histologic finding in varicocele testicular biopsies.[69–71]

SC responsiveness to follicle-stimulating hormone (FSH) may be diminished in varicocele patients. Stimulation of SCs with FSH reversed spermatogenesis arrest in experimental animal models.[72] Varicocelectomy restored normal levels of FSH.[73] Altered levels of serum inhibin found in patients with varicocele may reflect altered function of SCs.[74] Cameron and associates noticed that Sertoli-germ cell junctional complexes appeared to be structurally abnormal in patients with varicocele.[75] They concluded that testicular disruption associated with varicocele is a phenomenon of the adluminal compartment, and that SCs are more sensitive to perturbation of the testicular environment than are germ cells. SCs may be the primary intratubular site of alteration leading secondarily to spermatogenic disruption.[76] Histologic studies of the testis from patients with varicocele showed absent germ cells or altered spermatogonia to SC ratio, signs often associated with SC dysfunction.[77] In addition to measuring circulating hormone levels, screening for point mutations in the circulating hormones,

receptors, and secondary signaling systems may give more precise insight into male infertility and allow for patient stratification.[78]

Paracrine regulation of the testis. Insight into the detailed mechanism of spermatogenesis is even more complicated because it is also regulated by complex interactions and signals at the cellular level in the testis (Figure 3).[1,79]

Both SCs and LCs regulate spermatogenesis by steroidogenesis and growth factor production.[80,81] SCs tightly regulate germ cell proliferation and differentiation and are implicated in the control of germ cell apoptosis. Fas (APO-1, CD95), a transmembrane receptor protein expressed by germ cells, transmits an apoptotic signal within cells when bound by Fas ligand (FasL) produced by SCs. The Fas system has been implicated in regulation of apoptosis in germ cells.[82] SCs stimulated by FSH produce inhibin (In) and activin (Ac).[83] Inhibin has negative feedback control on the pituitary and FSH secretion. It also binds to LCs regulating T production. Activin binds to round spermatids and spermatogonia (SP), affecting spermatogenesis. SP are known to stimulate transferrin production by SCs by an unidentified protein substance.[84]

LCs control spermatogenesis not only by steroid production but also by epidermal growth factors (EGFs) that bind to SP and spermatids regulating cell divisions.[85] Receptors for transforming growth factor (TGF), one of the EGFs produced by LCs, are found in peritubular myoid cells (PC). PCs secrete peritubular myoid cell substance (PmodS) that stimulates SCs. LCs control adluminal tubular compartment and transport of nutrients from the vascular space to germinal epithelium by vascular endothelial growth factor (VEGF). VEGF is of particular interest in varicocele, because it regulates endothelial permeability and is an angioproliferative factor.[86]

Locally produced neurotropins play their distinct role in spermatogenesis regulation.[87] Opioid receptors are found on LCs. During stress, release of endorphins stimulates opioid receptors and decreases testosterone production. Blocking opioid receptors by naloxone restores testosterone production to normal.[88] Naloxone did not change the serum concentration of LH, FSH, or T when given to patients with varicocele.[89]

Free radicals may also be considered paracrine substances, because they are produced and act locally. There is strong evidence that varicocele is associated with increased concentration of ftee radicals in semen. Free radical concentrations normalize after varicocelectomy.[90,91]

With each discovery of a new paracrine substance and a better understanding of the molecular mechanisms controlling spermatogenesis, we will better define the pathophysiologic mechanisms responsible for growth arrest and deleterious effects on spermatogenesis. The ability to measure these substances may allow us to predict which adolescent and/or adult with varicocele requires surgical intervention.

Pathology

Testicular hypotrophy. The testicular function most affected by varicocele is spermatogenesis.[92] The most common findings on semen analysis are increased number of pathologic sperm forms, decreased motility, and decreased sperm density.[8,93,94] Sperm analysis in adolescents with varicocele shows decreased sperm density, increased number of pathological forms, and decreased motility; however, there are no established norms for adolescent semen analysis.[93] Varicocele is also associated with testicular growth arrest in adolescents.[10,12] Testicular growth arrest may be considered the hallmark of testicular damage in adolescent varicocele.

Testicular growth arrest may be considered the hallmark of testicular damage in adolescent varicocele.

Significant volume loss in adolescents with varicocele has been noted in 77% of boys, 10% of whom had a left testis one fourth the size of the right testis.[95] Testicular hypotrophy is time-dependent.[96,97]

Testicular volume during preadolescence is constant, and at the onset of puberty the testis suddenly increases in size even prior to other pubertal changes. In adolescents with varicocele, the rapid growth of the testis between the ages of 11 and 16 is affected by varicocele and results in a volume discrepancy between the right and left testis. The hypothesis that there is a negative correlation between testicular volume and sperm density, motility, and number of pathologic forms is well supported in the literature.[8,98,99]

Histopathology. Testicular biopsy in males with varicocele shows a wide array of abnormalities. The most common findings are LC hyperplasia, decreased number of SP per tubule, spermatogenesis arrest, and sloughing of germinal epithelium.[99-103] A thickened basement membrane of seminiferous tubules and proliferative lesions of endoepithelium are often demonstrated and may affect transport of oxygen and glucose through these structures.[104,105] The damage to the basement membrane seems to be time-dependent, as shown by ultra-

structural and immunohistochemical observations that highlighted focal damage at the level of the peritubular basal lamina in pubertal boys with varicocele, but this damage was not as severe as that described in adult varicocele.[106] Myofibroblasts obtained from pubertal males with varicocele showed no evidence of transformation into fibroblasts other than increased presence of extracellular matrix. In adult males, myofibroblasts transform into fibroblasts, with varicocele causing peritubular fibrosis. This can explain why the varicocelectomy in adolescents is able to reverse growth arrest and restore normal architecture of the testis, while the same procedure in adults with varicocele does not always affect testicular atrophy, although it may improve the semen characteristics.[107,108]

Peritubular fibrosis and increased thickness of basal membrane correlate negatively with sperm density and number of pathologic forms.[109] The longitudinal smooth muscle fibers in vein walls are hypertrophied in high-grade varicocele; however, it is not yet clear whether this is a cause or a consequence of varicocele. One can stipulate that vein wall hypertrophy may be the result of a high volume of blood flowing through the vessels or increased hydrostatic pressure.[110]

Diagnosis

The adolescent with varicocele is often asymptomatic, and the varicocele is detected on routine physical exam. The patient should be examined standing in a warm room to relax the scrotum and allow easier examination of the spermatic cord. The scrotum is first visually inspected for any obvious distention around the spermatic cord; a visible varicocele is considered a large or grade 3 varicocele. The scrotum, testes, and cord structures are then gently palpated. A palpable varicocele has been described as feeling like a bag of worms or a squishy tube. More subtle varicoceles may feel like a thickened, asymmetric cord. The nonvisible but palpable varicocele is considered to be moderate in size (grade 2). If a varicocele is not palpable, but the patient performs a Valsalva maneuver that distends the PP of veins, a small (grade 1) varicocele is present.

Secondary varicocele, especially on the right side, can be caused by serious conditions such as retroperitoneal tumors, kidney tumors, or lymphadenopathy.

After examining the patient in the standing position, the patient should be examined supine. Idiopathic varicocele is more prominent in the upright position and disappears in the supine position. A thickened cord due to a varicocele should resolve in the supine position, whereas a thickened cord due to a lipoma will not change when the patient is supine. Secondary varicocele, especially on the right side, can be caused by serious conditions such as retroperitoneal tumors, kidney tumors, or lymphadenopathy.[111] A secondary varicocele does not change its size so dramatically in the supine position.

Testicular size needs to be measured to determine whether the varicocele is adversely affecting the growth of the testis. The volume of a normal testis measures 1 cc to 2 cc in the prepubertal male. Because of extensive individual variation in normal growth and development, testicular size is correlated with Tanner stage, growth velocity, and bone age rather than chronological age.

A number of methods have been used to measure the size of the testis. These include visual comparison, rulers, calipers, comparative ovoids (Prader orchidometer), punched-out elliptical rings (Takahara orchidometer), and ultrasound. A high correlation (r = .992) between ultrasound and actual volume was noted and was shown to be highly reproducible.[112] The Prader orchidometer was shown to correlate with ultrasound measurement in 256 patients (r = .91), although the degree of correlation was dependent on the investigator's clinical experience. In a clinical study of 22 male adolescents with a varicocele, 24% of patients with growth arrest would have been missed if measured by Prader orchidometer alone, and 3 patients felt to have a significant size discrepancy (>2 mL) by Prader orchidometer measurements were found to be normal by ultrasound volume estimate.

Diamond and colleagues... recommend annual ultrasound assessment of testicular volume in adolescents with varicocele.

These findings indicate that clinical estimates of testicular size by the Prader orchidometer are not as accurate or reproducible as those determined by ultrasound. Accurate measurement is important because operative decisions rest in the balance.[113] This finding was recently confirmed by Diamond and colleagues who recommend annual ultrasound assessment of testicular volume in adolescents with varicocele.[114]

There is considerable disagreement as to what constitutes a significant size discrepancy justifying surgical intervention. Testicular ultrasound is the most accurate and reproducible method to assess testicular volume and significant testicular size variations. A volume difference of less than 2 mL can be due to the measurement technique alone. Therefore, size variation of more than 2 mL by ultrasound is currently the best indicator of testicular damage and should serve as the minimal requirement for surgical repair of the adolescent varicocele.[1] Surgical intervention reverses testicular growth arrest, and assessment of testicular volume postoperatively predicts resolution of the varicocele.[12,11]

It is hard to discount the association between varicocele and infertility in this era of common accessibility to the medical literature on the Internet.

Management

There are some cardinal questions to be answered regarding management of adolescent varicocele:

Once a varicocele has been found, what information needs to be given to the patient and his parents? A number of psychological reactions (anxiety, depressed mood) were experienced in approximately 30% of boys who were informed about varicocele.[116] Because the word *infertility* is often associated with sexual impairment, we believe that during discussion with the patient and his parents, the only fact that should be stressed is that varicocele may result in a decrease in testicular volume that can be reversed by surgical treatment. However, it is hard to discount the association between varicocele and infertility in this era of common accessibility to the medical literature on the Internet (we found 1743 Web pages in searching for the term *varicocele*; many included the word *infertility* in the text). Because there are studies that demonstrate an abnormal semen analysis in adolescents, it seems advisable to discuss all findings first with the parents who can be helpful in presenting the problem to the child.[117,118]

Once diagnosed, whom should we treat? Varicocele is the most common correctable cause of male infertility.[119] Two thirds of patients will have improvement in semen analysis after varicocele repair, and 40% of partners will become pregnant.[16] Historically, adolescent varicocele was left untreated because its relationship to infertility was not well established. Subsequently, Kass and Belman showed that testicular growth arrest could be reversed by varicocelectomy in adolescents.[120] Repair of varicocele not only reversed growth arrest but also improved semen analysis in adolescents and young males.[118,121,122]

There is good evidence that, if left untreated, with time the varicocele will continue to affect testicular growth with loss of volume and progressive deterioration in semen analysis.[6,123] Goldstein and Gorelick suggest that varicocele causes a progressive decline in fertility and that prior fertility in men with varicocele does not predict resistance to varicocele-induced infertility in the future, so-called *secondary infertility*.[6] However, Lund and Larsen followed 39 men with varicocele for 8 years and were not able to show progressive decline in semen analysis results compared with healthy subjects.[124]

The association between varicocele and infertility is well established.[7] The most difficult question is which clinical test should we use to establish the indications for surgical treatment in an adolescent with varicocele?

Currently, the clinical tests used to establish criteria for varicocele repair are:

1. Grade of varicocele
2. Measurement of testicular volume to assess testicular growth arrest
3. GnRH stimulation test
4. Measurement of PP vein diameter
5. Serum LH, FSH, and inhibin levels
6. Semen analysis

Varicocele is associated with testicular growth arrest in adolescents, and varicocele repair results in testicular "catch-up" growth.

1. Varicocele grade. Varicocele grade does not correlate well with abnormal semen analysis or infertility in adults.[125] There are varying opinions regarding correlation between grade of varicocele and degree of testicular hypotrophy in adolescents. Lyon and associates found no correlation of varicocele grade and testicular size in 30 adolescents.[95] In contrast, Skoog, Steeno, and Paduch all independently noticed that boys with severe varicocele have a smaller ipsilateral testicle.[10,113,116] It was also noticed that the smaller the testis, the worse the semen analysis results.[8,93] However, grade of varico-

cele by itself should not be the sole indication for treatment.

2. Testicular volume. There is an abundance of literature confirming that varicocele is associated with testicular growth arrest in adolescents, and varicocele repair results in testicular "catch-up" growth.[10,118,121,122] Testicular growth arrest with a volume difference of more than 2 mL assessed by ultrasonography is the most common indication for treatment.[1,10] The development of secondary infertility is another strong argument for early varicocele repair because, if left untreated, varicocele can not only affect testicular volume but also spermatogenesis.[6] A decrease in testicular volume is the best indicator for surgical correction of varicocele. However, not every boy with varicocele and testicular growth arrest will be infertile, and there is still a need to search for a test that would better distinguish between adolescents with a varicocele who will develop infertility and those who will remain fertile. In adult males, the situation is a little simpler because the indications for surgery are usually established after 12 months of infertility confirmed by abnormal semen analysis and the presence of varicocele. Obtaining a semen sample in adolescents is possible but difficult.

3. GnRH stimulation test. Damage to germinal epithelium results in compensatory stimulation of the pituitary gland and subsequent increase in FSH and LH production by gonadotrophs.[126] Intravenous administration of GnRH stimulates the pituitary gland to release FSH and LH. FSH levels are elevated in any condition (like varicocele) affecting the integrity of germinal epithelium.[127] In theory, the GnRH stimulation test could distinguish between adolescents with varicocele who have abnormal testicular functions and those who have normal spermatogenesis.[11] However, in clinical practice, the GnRH stimulation test is expensive, requires multiple serum samples, and lacks an association between abnormal results, growth arrest, and infertility.[128-130] An abnormal GnRH stimulation test was found in 30% of adolescents with varicocele and was not correlated with atrophy or infertility.[131] Currently, it seems that the GnRH stimulation test has a limited role in the clinical evaluation of adolescents with varicocele in the United States, but is still used in clinical trials in Europe.[132]

4. PP vein diameter. Ultrasonographic measurement of PP vein diameter (PPVD) has been used to look for subclinical varicocele in the adult infertile population when the physical exam is inconclusive and to follow persistent varicocele, but PPVD measurements are not useful in adolescents.[133,134]

5a. Inhibin level. Serum inhibin levels reflect the integrity of the seminiferous tubule and function of the SCs. Inhibin measurement in serum does not add any diagnostic information not found by FSH measurement. Inhibin B levels after dynamic GnRH stimulation failed to aid in stratification of adolescents with varicocele.[136] At present, there are not enough data to support the use of serum In levels in making clinical decisions.[74,83,137-139]

5b. FSH stimulation. FSH has been utilized as an adjuvant therapy after varicocelectomy in adult infertile patients; however, it also can be used in adolescents with varicocele. Radicioni and Schwarzenberg postulate that more than 50% of improvement in semen analysis in males treated with a 3-month course of FSH indicates a better outcome after varicocelectomy.[140] However, at present there isn't enough evidence to advocate FSH stimulation prior to varicocelectomy in adolescents.

6. Semen analysis. Increased number of pathologic forms, decreased progressive motility, and decreased sperm density are the most common abnormalities found on semen analysis in men with varicocele-related infertility. Varicocele is associated with impaired disposal of residual sperm cytoplasm in the testis and epididymis.[141] The retained sperm cytoplasm causes release of free radicals and infertility. Varicocelectomy was shown to lower the number of sperm with retained cytoplasm.[142] It is concluded that varicocele may cause deleterious alterations in early spermatid head differentiation during spermiogenesis and that varicocele patients with a high incidence of sperm acrosome and nucleus malformations are appropriate candidates for varicocele correction.[143] By applying strict morphologic criteria to semen analysis, it was shown that varicocele repair improves the morphologic abnormalities of the sperm head found on preoperative semen analysis.[144] Because there is good correlation among sperm morphology, fertility index, and results from artificial reproductive techniques (ART), it is prudent to offer varicocelectomy to men with abnormal sperm morphology even with normal testicular size.[145,146]

Management summary. Currently, prophylactic surgery for every adolescent with varicocele is not advised because it would result in the treatment of 15% of adolescents. However, it appears that treatment should be offered to:

1. Adolescents with testicular growth arrest (2 SD from normal testicular growth curves, more than 2 mL of difference between left and right testicles)
2. Adolescents with abnormal semen analysis with high-grade varicocele
3. Adolescents with symptoms: pain, heaviness, swelling
4. Adolescents with bilateral varicoceles

Table 2
Surgical Approach, Complications, and Relative Costs of Varicocele Repair

Technique	Hydrocele Rate	Failure Rate	Cost	References
Retroperitoneal	7%-10 %	9%-11% (artery sparing) <3% (artery taking)	Low	10,147 12
Inguinal	3%-7%	9%-12%	Low	150,163,188
Inguinal microscopic	<1%	2.1% 0.6%	Moderate	155 156
Laparoscopic	1.25%	9% 1.25% in adolescents	High	189 190
Embolization		19%	High	172

Treatment Options

Treatment options in the management of adolescents with varicocele originated from the practice in adult male infertility.

Surgical ligation and division of the testicular veins or intravenous embolization of the testicular veins accomplish varicocele repair.

Three open surgical approaches are currently used: subinguinal (Marmar), inguinal (Ivanissevich), and retroperitoneal (Palomo). Laparoscopic varicocele ligation is often used in adults. Embolization techniques, regardless of embolizing material, can be classified as antegrade (infusion through scrotal part of the PP veins) and retrograde (catheter placed through femoral vein puncture) techniques.

Failure rate, frequency of complications, cost, and outcome are important factors that need to be evaluated in choosing the preferable treatment option in adolescent patients (Table 2). It is important to remember that most studies on varicocele repair concern adult infertile males with varicocele and not adolescents.

Failure rate. Recurrence of the varicocele after the repair can occur in 9% to 16% of adolescents. It was shown by Lund and our group that persistence of varicocele should be assessed by performing an ultrasound 6 months after surgery.[148] Persistence of the varicocele results in lack of "catch-up" testicular growth.[12] Most authors attribute the high persistence rate to missed venous collaterals that run parallel to the main testicular vein. The collaterals can be quite difficult to identify and ligate separately from the testicular artery. The reported persistence rate using the artery-sparing retroperitoneal approach ranges from 3% to 11%.[12,147,149,150] Ligation of both testicular vein trunci and the artery has the advantage of a decreased

Main Points

- Varicocele is associated with time-dependent testicular growth arrest and is the most common correctible cause of male infertility.
- In adolescent males it is usually asymptomatic, on the left side, and diagnosed during routine physical examination.
- Testicular growth arrest with a volume difference of >2 mL assessed by ultrasonography is currently the best indicator of testicular damage and should serve as the minimal requirement for surgical repair of the adolescent varicocele.
- Varicocelectomy can reverse growth arrest in adolescents (and may prevent future infertility).
- Laparoscopic varicocelectomy should not be used in adolescents. Although the high retroperitoneal ligation of the testicular artery and veins is the treatment of choice in adolescents, pediatric urologists should consider using microscopic inguinal or subinguinal repair with arterial preservation.
- Clinical tests currently establish criteria for repair, and there is need for a test to distinguish between adolescents with varicocele who will develop infertility and those who will remain fertile.

persistence rate and does not result in testicular atrophy, because the testis has collateral arterial blood supply from the cremasteric and deferential arteries.[12,21] Atassi and colleagues achieved a persistence rate below 2% in adolescents treated by high retroperitoneal ligation with testicular artery ligation.[12] Artery and vein ligation using laparoscopy was also shown to result in similar catch-up growth of the testis in adolescents and a low persistence rate.[151] There is, however, some objection to simultaneous ligation of the testicular artery in men with previous inguinal surgery, as there may be a compromised blood supply from the cremasteric and deferential arteries. Interruption of the testicular artery in these patients creates a high probability of developing testicular atrophy. Subsequent vasectomy in patients with testicular artery division should be avoided because ligation of the vasal artery could result in testicular atrophy.

Nagar recently reported a low persistence rate after varicocelectomy, if patients were stratified using color Doppler ultrasound.[152] A high ligation was performed only when reverse blood flow was demonstrable in the varicocele during a Valsalva maneuver, and a low approach was utilized when this finding was absent. One can hypothesize that pelvic collateral and cross-communicating veins were responsible for the varicocele in patients who showed lack of reverse blood flow through the PP on ultrasound. Obviously, outcome in this group would be unsuccessful if managed by a high retroperitoneal approach. The high incidence of pelvic splanchnic veins communicating with the internal spermatic vein found during embolization confirms Nagar's hypothesis.[153] We previously showed the importance of cross-communicating veins and pelvic veins in the persistence of varicocele; these results and Nagar's observations are opening a whole new area for future study.[154]

We previously showed the importance of cross-communicating veins and pelvic veins in the persistence of varicocele; these results are opening a whole new area for future study.

The high varicocele persistence rate and postoperative hydrocele rate led to the development of microsurgical varicocelectomy. Both subinguinal and inguinal microsurgical repair are used quite often in adults with varicocele and indeed offer lower persistence rates and a low incidence of postoperative hydrocele.[155-157] The low persistence rate using the microsurgical inguinal repair is attributed to ligation of all distended veins and collaterals at the level of the internal inguinal ring.[155,156] Because more pediatric urologists and surgeons are gaining experience in microsurgical inguinal varicocele repair, we may see this procedure becoming a surgery of choice for adolescents.[158,159] Reports by Minevitch and Goldstein demonstrated a significantly lower rate of persistence and postoperative hydrocele in adolescent patients.[158,160,161] The microscopic approach allows one to ligate only the veins, leaving the lymphatic vessels intact and decreasing the postoperative hydrocele rate to less than 1%.[156,157] These encouraging reports were recently confirmed by a prospective study by Cayan and associates, who treated 468 patients by either high retroperitoneal ligation or inguinal microsurgical repair. Microsurgical varicocelectomy resulted in fewer cases of postoperative hydrocele and recurrences.[162]

Laparoscopic varicocele repair with and without artery-sparing modifications are suitable surgical techniques.[163-165] The testicular vein and artery can be divided with hemostatic clips or coagulated with bipolar electrode.[166] Laparoscopic varicocele ligation can be employed in adolescents.[167-169] Laparoscopic surgery in the pediatric population bares the risk of significant complications like bowel perforation, major vascular injury, pneumothorax, and incisional hernia. Laparoscopic varicocele ligation is more expensive, takes longer, and offers no benefits of pain management or earlier return to daily activities as compared with the microvascular inguinal approach.[170] In a prospective, randomized study to compare laparoscopy versus open repair for varicocele in adult males with infertility (160 patients in the laparoscopy arm and 120 in the inguinal arm), laparoscopy was shown to be more expensive and to lack any advantages over the inguinal approach.[171] We are also concerned about the much higher cost of laparoscopy and its potential risks. In our opinion, laparoscopic varicocelectomy should not be used

In a prospective, randomized study to compare laparoscopy versus open repair for varicocele in adult males with infertility, laparoscopy was shown to be more expensive and to lack any advantages over the inguinal approach.

in adolescents. A retroperitoneal approach or microsurgical inguinal repair is preferred.

Retrograde embolization, unfortunately is associated with an unacceptable high rate of persistence and is the most expensive of treatment techniques.[1] A possible explanation of such a high persistence rate of varicocele after embolization is the highly variable anatomy of the testicular venous drainage and technical difficulties.[172] Because the embolization is performed in otherwise healthy young males with approximately 70 more years of life, one should also be concerned about radiation exposure during embolization, with a subsequent 0.1% life-long risk for cancer.[173] Antegrade embolization is more often used to treat persistent varicocele than as an initial treatment.[174-178]

Other options to decrease the rate of varicocele persistence are intraoperative venography and methylene blue injections.[27,150,179,180] Intraoperative venography in theory should facilitate ligation of all testicular vein trunci and decrease the rate of persistence. Hart and associates recommend routine use of intraoperative venography because 16% of their 62 patients had missed venous vessels after initial venous ligation.[181] Similar conclusions, based on a decreased persistence rate, were also made by Levitt, Zaontz, and Gill.[182-184] However, Palmer and Kass reported no difference in their rate of varicocele persistence after repair with and without intraoperative venography.[147,150,179] Based on these studies, intraoperative venography offers a marginal benefit in prevention of persistent varicocele.

Based on our review of the literature and the results of a survey of pediatric urologists in the United States, high retroperitoneal ligation of the testicular artery and veins offers the best results in adolescents. Although currently the high retroperitoneal approach is a treatment of choice in adolescent varicocele, pediatric urologists should consider using the microscopic inguinal or subinguinal approach with arterial preservation.

The Future

What are future directions for urologists and scientists interested in varicocele? Advanced molecular biology techniques used for evaluation of the infertile male have increased our understanding of the physiology of spermatogenesis. We now realize that azoospermia and severe oligoasthenospermia are rare in pure varicocele. It is estimated that 14.7% of males with azoospermia carry microdeletions of the long arm of chromosome Y.[185] Males with varicocele and azoospermia or severe oligoasthenospermia may actually suffer from point mutation or deletion of genes important in spermatogenesis. It seems unlikely that those patients as either adults or adolescents will benefit from varicocelectomy. By screening for aberrations of genes involved in regulation of spermatogenesis, we will be able to exclude those with genetic abnormalities and establish better criteria for management of patients with varicocele.

Conclusions

The adolescent with varicocele presents the clinician with an interesting and challenging problem. There is a great need for further basic research to help better select patients who need surgical correction of varicocele. We have outlined recommendations that can be used in everyday practice. Each clinician needs to make his/her own decisions regarding appropriate candidates, timing, and methods for treatment of adolescents with varicocele. ■

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Asian Journal of Andrology (2012) 14, 676–682

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ORIGINAL ARTICLE

Deletion or underexpression of the Y-chromosome genes *CDY2* and *HSFY* is associated with maturation arrest in American men with nonobstructive azoospermia

Peter J Stahl, Anna N Mielnik, Christopher E Barbieri, Peter N Schlegel and Darius A Paduch

Maturation arrest (MA) refers to failure of germ cell development leading to clinical nonobstructive azoospermia. Although the azoospermic factor (AZF) region of the human Y chromosome is clearly implicated in some cases, thus far very little is known about which individual Y-chromosome genes are important for complete male germ cell development. We sought to identify single genes on the Y chromosome that may be implicated in the pathogenesis of nonobstructive azoospermia associated with MA in the American population. Genotype–phenotype analysis of 132 men with Y-chromosome microdeletions was performed. Protein-coding genes associated with MA were identified by visual analysis of a genotype–phenotype map. Genes associated with MA were selected as those genes within a segment of the Y chromosome that, when completely or partially deleted, were always associated with MA and absence of retrievable testicular sperm. Expression of each identified gene transcript was then measured with quantitative RT-PCR in testicular tissue from separate cohorts of patients with idiopathic MA and obstructive azoospermia. Ten candidate genes for association with MA were identified within an 8.4-Mb segment of the Y chromosome overlapping the AZFb region. *CDY2* and *HSFY* were the only identified genes for which differences in expression were observed between the MA and obstructive azoospermia cohorts. Men with obstructive azoospermia had 12-fold higher relative expression of *CDY2* transcript (1.33±0.40 *vs.* 0.11±0.04; *P*=0.0003) and 16-fold higher expression of *HSFY* transcript (0.78±0.32 *vs.* 0.05±0.02; *P*=0.0005) compared to men with MA. *CDY2* and *HSFY* were also underexpressed in patients with Sertoli cell only syndrome. These data indicate that *CDY2* and *HSFY* are located within a segment of the Y chromosome that is important for sperm maturation, and are underexpressed in testicular tissue derived from men with MA. These observations suggest that impairments in *CDY2* or *HSFY* expression could be implicated in the pathogenesis of MA.
Asian Journal of Andrology (2012) **14**, 676–682; doi:10.1038/aja.2012.55; published online 23 July 2012

Keywords: CDY1 protein; CDY2 protein; genetics; histology; HSFY; human; male infertility; nonobstructive azoospermia; spermatogenesis; sperm maturation

INTRODUCTION

Maturation arrest (MA) refers to the histological finding of germ cells throughout the seminiferous tubular epithelium that do not complete spermatogenesis. While it is believed that genetic abnormalities are more common in patients with MA than in men with other variants of nonobstructive azoospermia (NOA),[1] in the majority of patients, the cause of MA is not known. Many of these patients may have genetic anomalies that interfere with sperm production[2] which have not yet been discovered. Investigation of the genetic causes of MA is challenging due to several factors, including the absence of good animal models of MA, scarcity of human testicular tissue from men with MA for research and the likely polygenic nature of male infertility. Shotgun approaches to identification of genes important for completion of spermatogenesis with microarrays have been attempted using testicular tissue from men with NOA, but have been disappointingly unrevealing.[3]

The critical role of the Y chromosome in male fertility was first recognized by Tiepolo and Zuffardi[4] in 1976. One potential starting point in the search for genes associated with MA in humans, therefore, is the Y chromosome. Y-chromosome microdeletions involving complete or partial loss of the azoospermic factor (AZF) region are currently among the most common identifiable genetic causes of NOA, accounting for approximately 10% of cases.[5–7] Phenotypic characterization of infertile men with Y microdeletions provides insight into the spermatogenic function of genes within the deleted DNA segments, as men with Y microdeletions may be considered to be naturally occurring 'knockouts' for genes that have been deleted, most of which do not have functional autosomal homologues.

It is of particular interest that complete loss of the AZFb region causes MA and a poor prognosis for biological paternity.[5,8–10] This observation suggests that one or more genes within or surrounding AZFb may be critical for germ cell maturation, and that perturbation of expression of genes within AZFb or their function may underlie some cases of otherwise unexplained NOA associated with MA. However, despite our relatively advanced understanding of the genetic mechanisms and clinical sequelae of Y microdeletions, we still do not know which AZFb genes are essential for sperm production.

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Received: 21 March 2012; Revised: 20 April 2012; Accepted: 26 April 2012; Published online: 23 July 2012

In the present study, our objective was to identify Y-chromosome genes that may be implicated in the pathogenesis of idiopathic NOA associated with MA. Two strategies were implemented. First, we used genotype–phenotype analysis of phenotypically well-characterized infertile men with precisely mapped Y-chromosome microdeletions to identify candidate Y-chromosome genes that may be associated with MA. Next, we investigated whether or not we could detect differences in RNA expression of the identified candidate genes in testicular tissue from patients with MA and negative Y microdeletion testing. Other groups have successfully used this approach for investigation of some Y-chromosome genes in patients with NOA,[11–14] though prior studies have not focused on MA.

MATERIALS AND METHODS

Patient selection for genotype–phenotype analysis

The Institutional Review Board of the Weill Cornell Medical College approved this study. The study population was derived from a cohort of 246 men with Y-chromosome microdeletions who were identified by consecutive screening of 1997 infertile men in our laboratory for Y-chromosome microdeletions. The ethnicity distribution in the screened population was representative of the population of the United States, including 69% Caucasians, 23% African American, 6% Asian, 1% native Hawaiian or Pacific Islander and 1% native American. One hundred and thirty-two patients with Y microdeletions met inclusion criteria for the genotype–phenotype analysis. All non-azoospermic patients for whom semen analysis results were available were included. For azoospermic patients, only patients who underwent simultaneous cytological evaluation by microdissection testicular sperm extraction (TESE) and histological evaluation by diagnostic testicular biopsy were included.

Microdissection TESE is a surgical technique for sperm retrieval in men with NOA whereby all seminiferous tubules can be visually examined with an operating microscope. Dozens of samples (biopsies) are taken during the procedure and cytologically examined for sperm as well as germ cells. A random biopsy is also taken for histology, allowing complete and detailed phenotypic classification of the typically heterogeneous testes. This approach minimizes the risk of phenotypic misclassification due to sampling error that is inherent to random testicular biopsy. Azoospermic patients who did not undergo microdissection TESE for therapeutic purposes were excluded.

Y microdeletion testing

Y microdeletion testing was performed by multiplex PCR of DNA extracted from peripheral blood leukocytes. For each patient, genomic DNA was extracted from peripheral blood using two commercially available DNA extraction kits. Thirty sequence-tagged sites (STSs) within the AZF region of Yq11 and the *SRY* gene (sY14) were targeted for PCR amplification using previously published primer sequences.[15–17] All patients were tested twice with multiplex PCR using DNA extracted with each method. DNA from a fertile male served as a positive control. Water and DNA from a female were used as negative controls. Single-primer PCR analyses were performed in duplicate for all deleted STSs and two flanking STSs to confirm multiplex PCR results that indicated a Y microdeletion. STS amplification patterns that reflect AZFa, AZFb, AZFb+c and AZFc microdeletions are indicated in **Figure 1**.

Microdissection TESE and testicular biopsy

Azoospermia was confirmed on the day of sperm retrieval by microscopic analysis of ejaculated semen after centrifugation. Microdissection TESE was performed utilizing the operating microscope and a transverse incision in the tunica albuginea until sperm were found or the entire volume of testicular tissue was dissected.[18] Extracted testicular tissue was cytologically examined for the presence of sperm by an experienced andrologist in the operating room and subsequently in the andrology laboratory. Microdissection TESE was considered successful if one or more sperm were found that were morphologically acceptable for intracytoplasmic sperm injection.

Tissue acquisition for histopathology and RT-PCR

Diagnostic testicular biopsies and seminiferous tubular tissue for research were taken during microdissection TESE after the tunica albuginea was widely opened. Randomly selected pieces of undisturbed seminiferous tubular tissue measuring 5–10 mm in greatest dimension were sharply excised. One piece of tissue was placed gently into Bouin's solution for pathological analysis. Tissue for research was placed without media into a cryovial, immediately snap frozen in liquid nitrogen and stored at −80 °C.

Pathological analysis of testicular biopsies

Histopathological analysis was performed as previously described.[19] Sections were stained with hematoxylin and eosin and examined with a light microscope under ×100 to ×400 magnification. Biopsies were classified according to the most advanced pattern of spermatogenesis observed anywhere within the tissue biopsied. We classified biopsies as Sertoli cell only (SCO) when germ cells were completely absent ('pure SCO'), and as MA when germ cells were identified anywhere in the biopsy specimen but oval sperm heads were completely absent (**Figure 2**). For example, a biopsy that was comprised of 95% SCO pattern and rare tubules containing spermatocytes was classified as MA, not SCO.

Phenotypic characterization

Combined results of semen analyses, diagnostic testicular biopsies and microdissection TESE were used to classify patients with Y microdeletions by testicular histopathological phenotype. Patients were classified as either capable or incapable of mature sperm production. The 'capable of mature sperm production' group included oligozoospermic patients and those for whom spermatozoa were identified on testicular biopsy or in tissue extracted during microdissection TESE. Therefore, a man with sperm production so poor that sperm were not present in the ejaculated semen sample but could only be found in focal areas of the testes would be classified as 'capable of mature sperm production' despite his quantitatively very impaired production. This group included patients with histological hypospermatogenesis, as well as patients with diagnostic biopsies that showed SCO or MA but in whom sperm were successfully retrieved. Azoospermic patients in whom microdissection TESE failed were considered incapable of mature sperm production. This cohort was subclassified based on the most advanced spermatogenic pattern evident on testicular biopsy as either SCO or MA.

Genotype–phenotype analysis

A genotype–phenotype map was constructed to enable visual analysis of genotype–phenotype correlations (**Figure 1**). Patients were placed into three histopathological phenotypic categories: (i) incapable of mature sperm production/SCO; (ii) incapable of mature sperm production/MA; and (iii) capable of mature sperm production. The Y microdeletion of each patient was mapped according to its STS amplification pattern and the published sequence of the Y chromosome

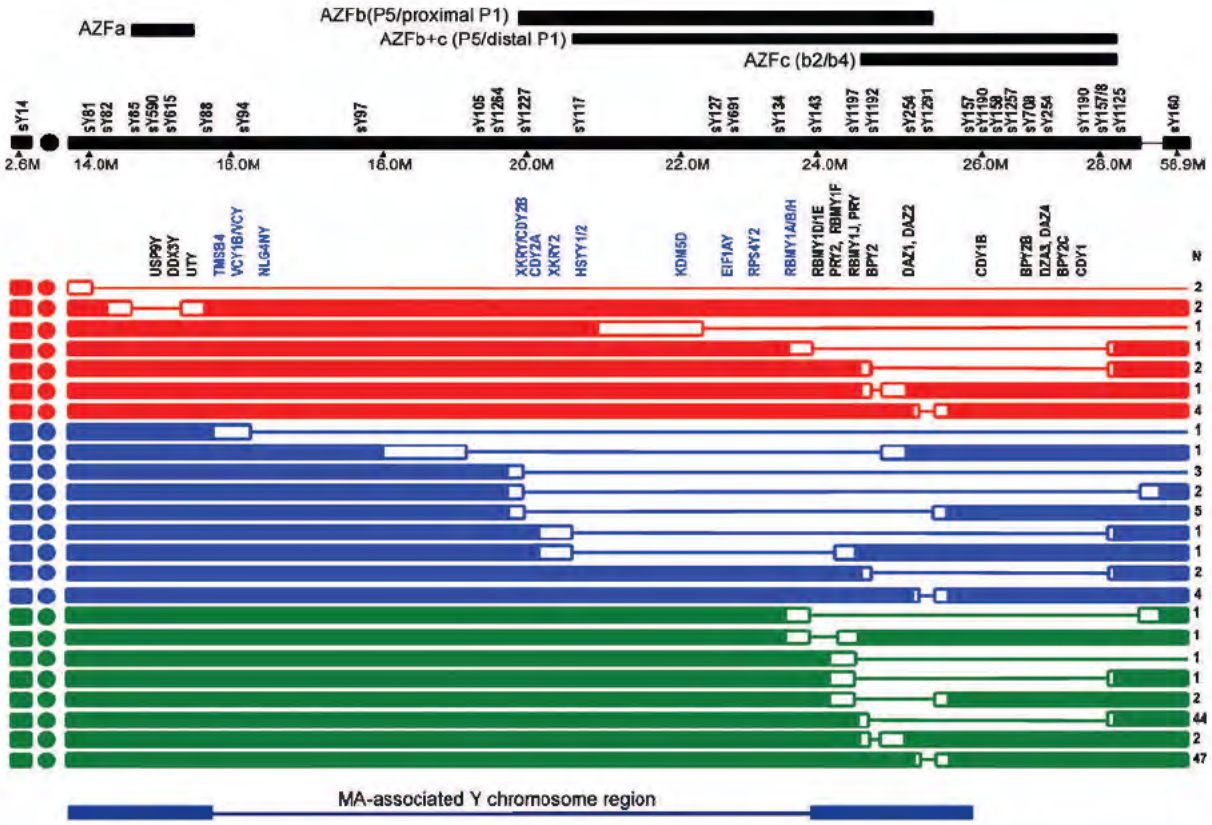

Figure 1 Genotype phenotype map constructed to enable visual analysis of genotype phenotype correlations. STSs used for Y microdeletion screening in our laboratory and the protein-coding genes within the AZF region are indicated in their respective positions on Yq11. Black bars indicate the STS amplification patterns used in our laboratory to diagnose the AZFa, AZFb (P5/proximal P1), AZFb+c (P5/distal P1) and AZFc (b2/b4) Y microdeletions. Y microdeletion breakpoints based upon STS amplification patterns observed during Y microdeletion testing are indicated for the 132 patients with Y microdeletions analyzed. Patients were placed into one of three phenotypic categories for mapping: (i) incapable of mature sperm production/SCO histology (red color), (ii) incapable of mature sperm production/MA histology (blue color), and (iii) capable of mature sperm production (green color). The number of patients with each Y microdeletion pattern depicted is indicated on the far right. Solid bar, STS presence; thin line, STS absence; hollow bar, DNA segment located between STSs for which there is insufficient information to determine DNA segment presence or absence. The AZF segment that we identified as associated with MA is indicated at the bottom of the map. AZF, azoospermic factor; MA, maturation arrest; SCO, Sertoli cell only; STS, sequence-tagged site.

(http://www.ncbi.nlm.nih.gov/projects/mapview/map_search.cgi?taxid=9606).

The region of the Y chromosome associated with MA was identified by visual analysis of the genotype–phenotype map by the following criteria: (i) immature germ cells were histologically identified in the majority of patients with complete or partial deletions of the region; and (ii) all patients with complete or partial deletions of the region were incapable of sperm production (i.e., ejaculated or testicular sperm were never found in any patient in whom the region was completely or partially deleted). The borders of the MA-associated region were defined by the nearest flanking nondeleted STSs. The protein-coding genes within the MA-associated region of the Y chromosome were determined by online query of the NCBI HuRef-primary assembly database (http://www.ncbi.nlm.nih.gov/projects/mapview/map_search.cgi?taxid=9606).

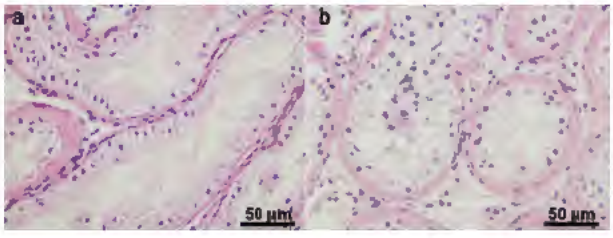

Figure 2 Representative testicular biopsies from patients with idiopathic NOA and failed microdissection TESE. Hematoxylin and eosin staining. (**a**) SCO pattern. (**b**) MA pattern at the level of the pachytene spermatocyte. Rare cells with condensed nuclei are present and may represent apoptotic germ cells or early spermatids. MA, maturation arrest; NOA, nonobstructive azoospermia; SCO, Sertoli cell only; TESE, testicular sperm extraction.

Selection of patients with idiopathic NOA for RNA expression analysis

We queried our prospectively maintained database of NOA patients who underwent microdissection TESE and simultaneous diagnostic testicular biopsy. We identified patients with idiopathic NOA associated with MA or SCO histology who had normal 46XY karyotypes, negative Y microdeletion testing and absence of any identifiable clinical factors associated with infertility such as prior orchitis, cryptorchidism, hypogonadotropic hypogonadism and chemotherapy or radiation exposure. Testicular sperm were not retrieved by microdissection TESE in any of the included patients. Tissue for research was available for 19 patients with NOA associated with MA, 13 patients with NOA associated with 'pure' SCO and eight patients with OA (who have normal sperm production and served as controls). The

MA group included 11 patients with early MA, in whom germ cell development was arrested at the primary spermatocyte or spermatogonial stages, and eight patients with late MA, in whom germ cell development was arrested at the stage of the round spermatid. Additionally, RNA expression of all candidate genes studied was analyzed in testicular tissue from one patient with an AZFb deletion.

RNA extraction and RT-PCR

Snap-frozen seminiferous tubular tissue for RNA extraction was thawed, weighed and homogenized. RNA was extracted with a commercially available monophasic solution of phenol and guanidine isothiocyanate (Life Technologies, Carlsbad, CA, USA). To remove contamination with genomic DNA, extracted RNA was incubated with RNase-free DNase (Qiagen, Hilden, Germany) for 30 min purified with a commercially available RNA-binding spin column (Qiagen). RNA concentration was measured spectrophotometrically at 260 nm and the purity was confirmed by measurement of the A260/A280 ratio. cDNA was synthesized from 1 μg of purified total RNA with random hexamer primers and stored at −20 °C until use.

Candidate gene mRNA level was measured using dual-color, multiplex qRT-PCR with the Universal Probe Library (UPL) hydrolysis probe set on a LightCycler 480 instrument (Roche Diagnostics Corp., Basel, Switzerland). *PBGD*, a gene encoding porphobilinogen deaminase was used as the housekeeping gene for relative quantification based upon observations in our laboratory of consistent *PBGD* RNA expression in human testis irrespective of histology (data not shown). The multiplex assays were designed using the UPL Assay Design Center (http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp). Primer sets and detection probes for each candidate gene are indicated in **Table 1**. *PBGD* mRNA was detected with a proprietary Human *PBGD* Gene Assay (Roche Diagnostics Corp.).

All qRT-PCR reactions were run in duplicate on 96-well plates. The 20-μl reaction mixture contained 5 μl of 1:5 diluted cDNA and 200 nmol l^{-1} UPL probe, 200 nmol l^{-1} *PBGD* probe, 200 nmol l^{-1} forward and reverse primers for *HSFY*, 500 nmol l^{-1} forward and reverse primers for *PBGD* and ×1 LightCycler 480 Probes Master mix. The cycle protocol used was: denaturation at 95 °C for 10 min, 45 cycles of 95 °C for 10 s and 60 °C for 30 s, and a cooling cycle to 55 °C with single fluorescence acquisitions at the end of each cycle. Candidate gene/*PBGD* expression ratios were determined with LightCycler 480 Relative Quantification software (Roche Diagnostics Corp.) using crossing points that were determined by the second derivate maximum method and standard curves that were generated during each PCR run for both the candidate gene and *PBGD*. Standard curves were generated by running the multiplex reactions in triplicate with serially diluted cDNA from a patient with OA. PCR-efficiency corrections and color compensation were applied by the software based on the standard curves and the calculated efficiencies for each candidate gene and *PBGD*.

Statistical analysis

Statistical differences in candidate gene expression between patients with MA and OA were assessed by the Mann–Whitney test. Two-tailed *P* values less than 0.05 were considered statistically significant. For the candidate genes with observed differences in expression between the MA and OA cohorts, one-way analysis of variance with Bonferroni post-test analysis was performed to investigate differences in expression between patients with SCO, early MA, late MA and OA.

RESULTS

Results of the genotype–phenotype analysis are depicted in **Figure 1**. We identified an 8.4-Mb DNA segment as a Y-chromosome region containing candidate genes for association with MA. This region spans the 4.4 Mb centromeric to the AZFb region and the centromeric 4.0 Mb of the AZFb region itself (from the centromeric border of AZFb to sY143). This region was completely or partially deleted in 15 patients, all of whom were classified as incapable of mature sperm production (azoospermic with absence of sperm upon bilateral microdissection TESE). Fourteen of the patients with deletions involving this region had MA histology and one had SCO. In contrast, we found complete sperm production in six patients with deletions involving the telomeric 1.6 Mb of the AZFb region (from sY143 to the telomeric border of AZFb).

This region of the Y chromosome that was identified as associated with MA contains 10 protein-coding genes (*TMSB4*, *VCY*, *NLGN4Y*, *XKRY*, *CDY2*, *HSFY*, *KDM5D*, *EIF1AY*, *RPS4Y2* and *RBMY*) that were designated as candidate genes for association with MA. Of these 10 genes, only *RBMY* exists in additional copies outside of this AZF segment (multiple *RBMY* copies are present within the AZFc region). We analyzed testicular RNA levels for eight of the 10 identified candidate genes. We did not study *XKRY* because we could not design a valid qRT-PCR assay, and we elected not to study *RBMY*, because it exists in multiple copies outside of the Y-chromosome region of interest.

The clinical characteristics of the patients with idiopathic MA and OA included in the transcript expression analysis are presented in **Table 2**. The relative transcript expression of each candidate gene was evaluated in testicular tissue derived from one patient with an AZFb deletion. The transcript ratios were negligible for each of the genes located within the known deleted interval in this patient

Table 1 Primers and UPL probes used in dual-color, multiplex real-time PCR assays for detection of candidate gene RNA transcript expression

Gene	*Forward primer*	*Reverse primer*	*UPL number*
TMSB4	5′-tgctccctacggcttcct-3′	5′-cttgcctctcctgttcgatag-3′	46
VCY[a]	5′-ggccaaggagacaggaaag-3′	5′-cggccaccttggtagtctt-3′	45
CDY2[b]	5′-ggcgaaagctgacagcac-3′	5′-gggtgaaagttccagtcaaaa-3′	54
NLG4NY	5′-aagaacgacgtcatgctcagt-3′	5′-tggttggtttggatcacca-3′	9
EIF1AY	5′-catgctaaaatcaatgaaacagaca-3′	5′-tgtcatgtaaaaacacttggttca-3′	80
HSFY[b,c]	5′-gtcaatgaggctccatatcgt-3′	5′-gatcgtaggcatttgcaacc-3′	40
KDM5D	5′-tctggagccaaccatgtg-3′	5′-gaaggctgcacagactgtctaa-3′	89
RPS4Y2	5′-tgggaagataaccagctttatca-3′	5′-accaggatgtctttccctgtt-3′	67

Abbreviation: UPL, Universal Probe Library.
[a] *VCY* primers targeted both transcripts.
[b] Nonintron spanning assays.
[c] *HSFY* primers targeted transcript variant 1.

Table 2 Clinical characteristics of patients included in the testicular RNA transcript expression analyses[a]

	MA (n=19)	OA (n=8)	P-value (MA vs. OA)
Age (year)	33.7±6.3	37.2±7.7	NS
FSH (IU/l)	12.8±11.6	3.7±2.2	0.04
Average testicular volume (ml)	12.8±4.4	13.9±3.7	NS

Abbreviations: FSH, follicle-stimulating hormone; MA, maturation arrest; NS, not statistically significant; OA, obstructive azoospermia.
[a] Data given as mean±s.d.

(Figure 3). We observed differences in expression between the MA and OA cohorts for *CDY2* and *HSFY*. Men with OA had 12-fold higher relative expression of *CDY2* transcript (1.33±0.40 *vs.* 0.11±0.04; P=0.0003) and 16-fold higher expression of *HSFY* transcript (0.77±0.32 *vs.* 0.05±0.02; P=0.0005) compared to men with MA. We did not observe significant differences in testicular transcript expression ratios for any of the other candidate genes (**Table 3** and **Figure 4**).

CDY2 and *HSFY* expressions were further analyzed with respect to testicular histopathological phenotype by comparison of gene/*PBGD* transcript ratios in testicular tissue derived from patients with NOA associated with SCO histology, NOA associated with early MA, NOA associated with late MA, and OA. Both *HSFY* (P<0.0001) and *CDY2* (P<0.0001) were significantly underexpressed in tissue derived from SCO patients (P<0.0001 and P<0.001, respectively) when compared to tissue derived from patients with OA. We did not observe significant differences in expression between the SCO, early MA and late MA cohorts (**Table 4** and **Figure 5**).

DISCUSSION

Better understanding of the molecular mechanisms that regulate spermatogenesis and the genetic disturbances that cause MA will allow us to develop novel diagnostic tests and may ultimately lead to more forms of therapy in the future. We elected to focus our search for candidate MA genes on the Y chromosome, which has a well-established but incompletely understood role in germ cell maturation. Thus far, no single Y-chromosome gene has been definitively linked with MA.

Table 3 Expression of each candidate gene RNA transcript relative to expression of the housekeeping gene ***PBGD*** in patients with MA and OA[a]

Gene/PBGD transcript expression ratio	MA	OA	P-value
TMSB4	0.99±0.16	0.84±0.11	0.62
VCY	0.70±0.12	0.35±0.05	0.17
NLG4NY	0.96±0.28	0.87±0.29	1.00
CDY2	0.11±0.04	1.33±0.40	0.0003
HSFY	0.05±0.02	0.77±0.32	0.0005
KDM5D	6.50±0.60	5.06±1.69	0.09
EIF1AY	2.26±0.59	3.19±049	0.44
RPS4Y2	2.84±0.39	2.38±0.43	0.62

Abbreviations: MA, maturation arrest; OA, obstructive azoospermia; PBGD, porphobilinogen deaminase.
[a] Data given as mean±s.e.

Deletions of the AZF region genes, such as occurs in Y microdeletions, cause multiple downstream effects on the testicular transcriptome involving altered expression of hundreds of genes.[20] This observation suggests a central, regulatory role of the AZF gene products in spermatogenesis. Mutations of genes in the AZF regions themselves or in genes that regulate their expression would be expected to have significant downstream consequences. Such genetic alterations would not be detectable by Y microdeletion testing and might underlie same cases of MA that are presently considered to be idiopathic. We identified an 8.4-Mb region including the 4.4-Mb DNA segment centromeric to the AZFb region and the centromeric 4.0 Mb of AZFb as the Y-chromosome region containing genes that may be associated with MA. Our genotype–phenotype analysis confirms the well-established importance of the AZFb region for germ cell maturation, and adds to the present understanding of AZFb by suggesting that the centromeric 4.0-Mb section of this region (from the centromeric border of AZFb to sY143) is the critical AZFb subregion.

Interestingly, we found complete sperm production in six patients with deletions involving the telomeric 1.6 Mb of the AZFb region (from sY143 to the telomeric border of AZFb), suggesting that this segment of AZFb is nonessential for sperm production. This nonessential segment contains both copies of the *PRY* gene and four of the

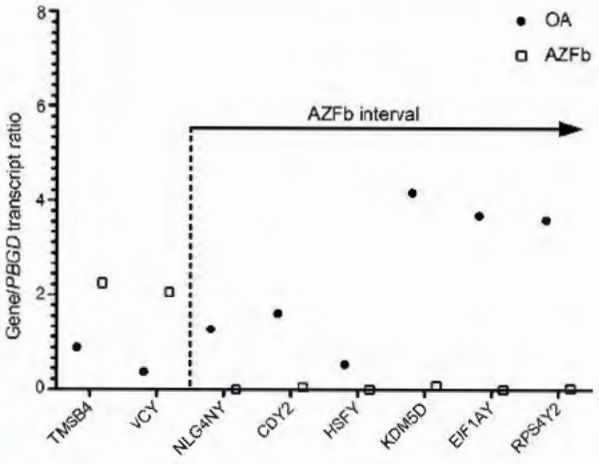

Figure 3 Relative testicular RNA transcript expression of each candidate gene for MA in one patient with an AZFb deletion, and in a representative patient with OA. The genes within the known deleted AZFb interval are indicated. AZF, azoospermic factor; MA, maturation arrest; OA, obstructive azoospermia; PBGD, porphobilinogen deaminase.

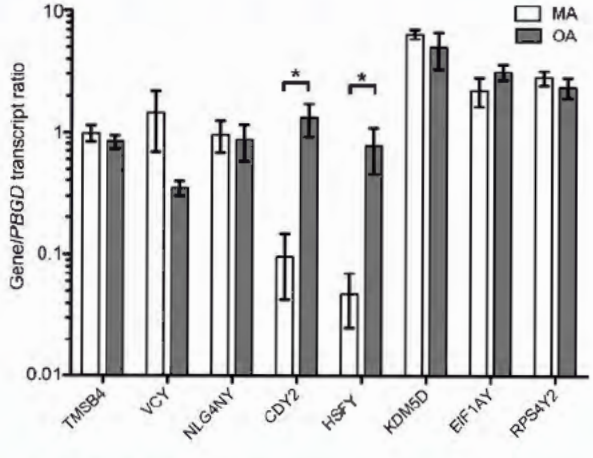

Figure 4 Relative testicular RNA transcript expression of each candidate gene for MA in patients with idiopathic NOA due to MA, and in patients with OA. Gene expression is normalized to expression of the housekeeping gene *PBGD*. *P<0.0001 on one-way ANOVA with Bonferroni *post hoc* analysis. MA, maturation arrest; NOA, nonobstructive azoospermia; OA, obstructive azoospermia; PBGD, porphobilinogen deaminase.

Table 4 Expression of each candidate gene RNA transcript relative to expression of the housekeeping gene *PBGD* in patients with SCO, early MA, late MA and OA[a]

Gene/PBGD transcript expression ratio	*SCO*	*Early MA*	*Late MA*	*OA*
CDY2	0.02±0.003	0.05±0.02	0.21±0.08	1.33±0.40*
HSFY	0.002±0.0004	0.06±0.04	0.03±0.02	0.77±0.32^#

Abbreviations: MA, maturation arrest; OA, obstructive azoospermia; PBGD, porphobilinogen deaminase; SCO, Sertoli cell only.
*$P<0.0001$ *vs.* SCO, early MA and late MA.
^$P<0.0001$ *vs.* SCO.
# $P<0.001$ *vs.* early MA and late MA.
[a] Data given as mean±s.e.

six copies of *RBMY*, demonstrating that PRY and four of the six copies of *RBMY* are not necessary for complete sperm production in all men. These findings are supported by a recent case report of a microdeletion involving this region that was naturally transmitted from father to son.[21]

The Y-chromosome region that we identified as associated with MA includes both copies of *CDY2* (*CDY2*A and *CDY2*B), which was one of two Y-chromosome genes found to be underexpressed in testicular tissue derived from men with NOA and MA. *CDY2* belongs to the family of human chromo domain proteins that includes two copies of *CDY1* within the more telomeric section of the AZFc region of the Y chromosome, as well as the autosomal genes *CDYL* and *CDYL2*. In contrast to the autosomal chromo domain proteins that are ubiquitously expressed in humans, *CDY2* and *CDY1* are exclusively expressed in testis.[22]

The function of the *CDY* genes in spermatogenesis remains incompletely understood. They encode proteins that contain both a chromatin-binding domain and a catalytic domain that is often found in acylation enzymes, suggesting that CDY proteins may interact with histones during chromatin remodeling.[22,23] Indeed, CDY proteins have been shown *in vitro* to exhibit histone acetyltransferase activity, and CDY protein expression has been localized in human testis to the nuclei of maturing spermatids.[12,24] However, the recently elucidated crystalline structure of CDY2 is surprisingly dissimilar from the structure of other known histone acetyltransferases.[25]

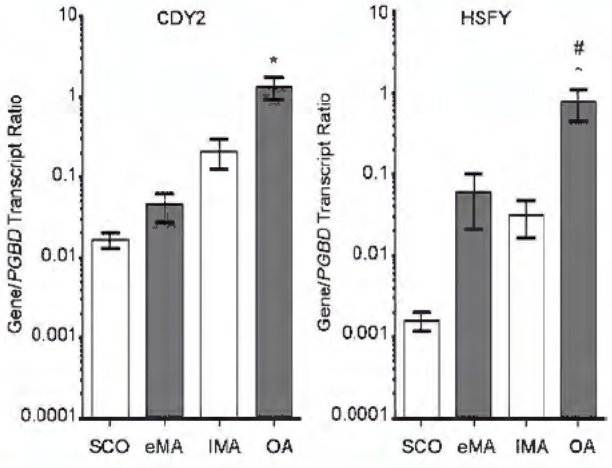

Figure 5 Relative testicular RNA transcript expression of CDY2 and HSFY in patients with histological variants of NOA (SCO, eMA and IMA) and OA. Gene expression is normalized to expression of the housekeeping gene *PBGD*. Statistical analysis between cohorts was performed with one-way ANOVA with Bonferroni *post hoc* analysis. *$P<0.0001$ compared with SCO, eMA and IMA. #$P<0.0001$ compared with SCO and ^$P<0.001$ compared with eMA and IMA. eMA, early maturation arrest; IMA, late maturation arrest; NOA, nonobstructive azoospermia; OA, obstructive azoospermia; PBGD, perphobilinogen deaminase; SCO, Sertoli cell only.

HSFY was the other RNA transcript associated with MA that was underexpressed in testicular tissue derived from men with idiopathic NOA due to MA. Two copies of *HSFY* are present within palindrome P4 of the AZFb region of the Y chromosome.[26] These genes encode three different mRNA transcripts that are expressed in human testis. The protein translated from mRNA transcript variant 1 contains a heat-shock factor-like DNA-binding domain,[27] suggesting that this mRNA is the critical *HSFY* transcript.

Though the function of *HSFY* is not presently understood, it is expressed in human germ cells and Sertoli cells[28] and likely acts by moderating expression of heat-shock proteins, which serve as important transcription factors. The DNA binding capacity of HSFY protein suggests a regulatory role for this gene during spermiogenesis. Decreased expression of HSFY protein has been associated with MA in humans,[29] and expression analysis of a mouse orthologue demonstrated predominant expression in round spermatids, supporting a role for *HSFY* in the later stages of spermatogenesis.[30] Our data suggest that *HSFY* RNA expression may play a critical role in germ cell maturation in the American population.

Interestingly, three oligozoospermic patients with identical partial AZFb deletions leading to isolated loss of *HSFY* have recently been reported.[31] This unique Y-chromosome microdeletion was detected in 3/1186 infertile men and 0/1179 control men, confirming that *HSFY* deletions are associated with infertility, but contradicting our findings that *HSFY* expression is essential for germ cell development. One plausible explanation for the different effects of *HSFY* loss in different populations is that X-linked or autosomal compensatory mechanisms may exist in some populations that can partially compensate for absence of *HSFY*. Indeed, all three of the reported oligozoospermic men with *HSFY* deletions belonged to the same specific Y-chromosome haplotype (R1b1b1a1b).

Although our data establish an association between underexpression of *CDY2* and *HSFY* and idiopathic NOA associated with MA, it would be premature to conclude that these genes play a causal role. Differential testicular expression of candidate gene mRNA in infertile patients with MA could reflect either a true pathogenic mechanism, or simply the absence of cell types to which candidate gene expression is normally localized. Our study (or any evaluation that compares gene expression with histology) cannot distinguish between these two competing explanations, which represents a significant limitation. Nonetheless, the observations reported in this study contribute to the present understanding of the function and expression of Y-chromosome genes in NOA associated with MA. These data have the potential to empower researchers and clinicians to improve the clinical care of patients with NOA. Even testicular microdissection, which is widely considered to be the most effective sperm retrieval

procedure available today, fails in 37%–57% of cases.[32] Further elucidation of *CDY2* and *HSFY* function and expression may lead to novel therapeutic or diagnostic approaches that could benefit patients with NOA.

AUTHOR CONTRIBUTIONS

PJS, PNS and DAP conceived and designed the study. PJS and ANM collected the data. PJS, CEB and DAP performed the statistical analysis. PJS, ANM, CEB, PNS and DAP drafted and revised the manuscript. All authors read and approved the final version.

COMPETING FINANCIAL INTERESTS

The authors have no competing financial interests.

ACKNOWLEDGMENTS

Dr. Peter Stahl is supported by the Research Scholar Award from the American Urological Association Foundation, and by private funding from the Frederick J. and Theresa Dow Wallace Fund of the New York Community Trust.

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Review Article

Diagnosis, evaluation and treatment of adolescent varicocele

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key words: adolescent varicocele, anatomy, pathophysiology, pathology, ultrasound evaluation, semen analysis, surgery, embolization

SUMMARY

A varicocele, an abnormal tortuosity and dilation of the veins of pampiniform plexus, appears in males at early puberty. There is an association between presence of varicocele, testicular growth arrest and infertility. Approximately 30–50% of males with primary infertility have a varicocele. Authors reminded anatomy, pathophysiology and pathology of the disease. The diagnostic methods, indications for therapy and treatment options were discussed next. Review of the current literature helped to provide guidelines for managing the adolescent with a varicocele. Although the need for treatment of adolescent varicocele in no longer controversial, authors stressed the necessity for further basic research in order to establish the best criteria to select the patients for surgery.

Med Sci Monit, 1999; 5(6): 1255-1267

INTRODUCTION

A varicocele can be defined as an abnormal tortuosity and dilation of the veins of the pampiniform plexus. Idiopathic varicocele is usually asymptomatic. It is noticed as an asymmetry in scrotal size, and presents as heaviness in the scrotum, or rarely with testicular pain. In most cases the adolescent is unaware of the varicocele and it is discovered during a regular physical examination or during examination for military service [1-4].

The incidence of high-grade varicocele is approximately 5% throughout the world [5]. Varicocele is associated with a time dependant testicular growth arrest in adolescents and adult males[6]. There is a clear association between varicocele, infertility and testicular growth arrest [7-9]. It is also known that varicocelectomy can reverse growth arrest in adolescents [10-13]. These facts have raised the question of how to best manage the adolescents with a varicocele.

Adolescents do not present with infertility and thus should prophylactic repair be performed to prevent infertility in the future? Who would benefit the most by varicocelectomy: the adolescents with testicular growth arrest or any adolescent with a varicocele? Is it better to wait for a semen analysis or offer earlier treatment based on testicular growth arrests? These questions can be only answered when we have better understanding of the pathophysiology of varicocele.

The purpose of this review is to present the current literature on adolescent varicocele and provide guidelines to the clinician how to manage the adolescents with a varicocele.

EPIDEMIOLOGY

In the general population of healthy males the overall incidence of varicocele (all grades) is 10% to 15% [4,5,14,15]. Approximately 30-50% of males with primary infertility have a varicocele [16-

Received: 99.09.22
Accepted: 99.11.10
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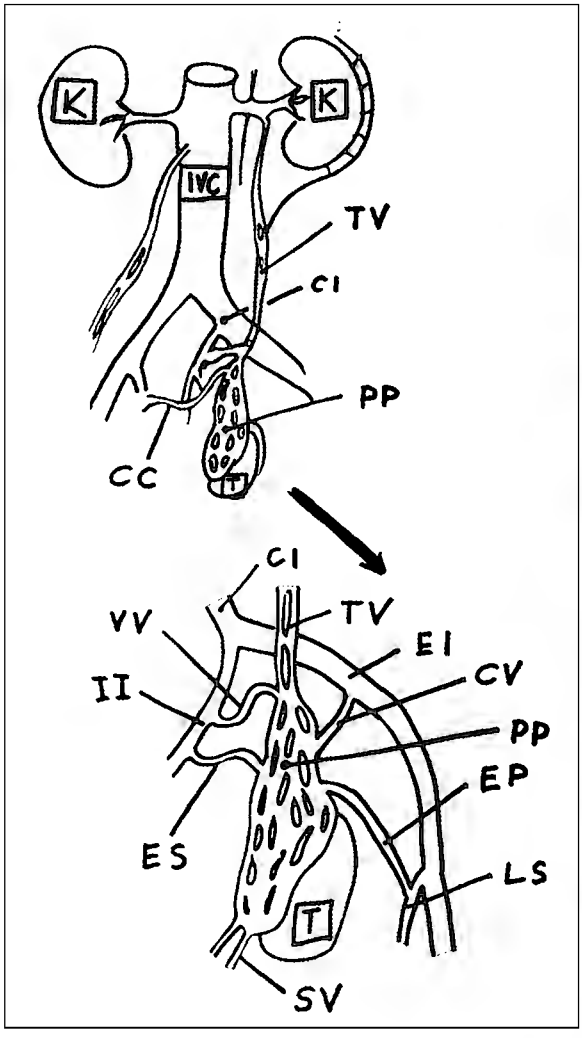
Table 1. Incidence of varicocele in general population of healthy adolescents.

Reference:	No of patients:	Age:	Incidence: (total)
Oster, 1971 [101]	837	10-19	16.2%
Steeno, 1976 [103]	4067	12-25	14.7%
Yerokhin, 1979 [146]	10000	10-17	12.4%
Belloli, 1993 [9]	9861	10-16	16.0%
Niedzielski, 1997 [5]	2478	10-20	17.8%

Figure 1. Anatomy of venous drainage from left testis: K – kidney, IVC – inferior vena cava, TV – testicular vein and trunci, CI – common iliac vein, CC – cross-communications, PP – pampiniform plexus, VV – vas vein, II – internal iliac vein, ES – external spermatic vein, SV – scrotal veins (gubernaculum), T – testis, LS – long saphenous vein, EP – external pudendal vein, EI – external iliac vein, CV – cremasteric vein.



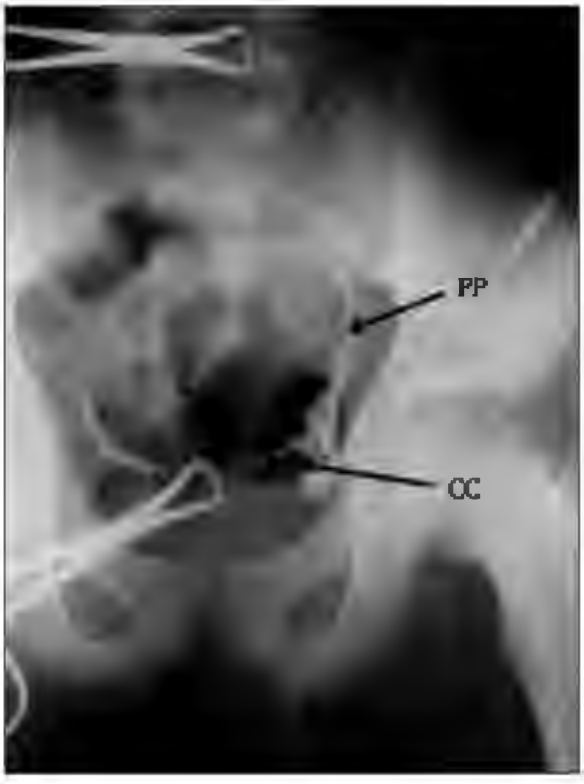
19]. Varicocele is most common on the left side. Varicocele appears at early puberty however it can occasionally be found in preadolescent boys [2,20]. The incidence in older adolescence varies from 12.4% to 17.8% with an average of 14.7%, similar to the incidence in adult males (Table 1).

ANATOMY

The arterial blood supply to the testis comes from the testicular artery, vasal artery and the cremasteric artery. At the level of testis all three arteries anastomose to allow adequate blood supply even with division of the testicular artery [21,22].

The venous drainage (Figure 1) is more complicated with many individual variations. Above the testis is a network of communicated veins called the pampiniform plexus (PP), the drainage from PP is via the testicular vein trunci, pudendal veins and cremasteric veins [23,24]. In most cases the testicular vein trunci form a single testicular vein entering the renal vein on the left and the inferior vena cava on the right. Venographic studies have demonstrated that the left testicular vein can rarely enter the

Figure 2. Intraoperative venogram showing left to right cross-communicating veins: CC – cross-communications, PP – pampiniform plexus veins.



inferior vena cava, and there are communications between the testicular vein and the inferior vena cava below the level of the renal veins [25-27].

There are also cross-communications between the left and right testicular venous systems (Figure 2) [26,28-30].

ETIOLOGY

There are several theories attempting to explain the etiology of varicocele. The predominance of the left side varicocele and the unique anatomy of the left testicular vein are the basis for several theories explaining the etiology of varicocele.

The presence of venous valves was long believed to be a guarding mechanism against developing a varicocele and incompetence of the venous valve system was thought to be responsible for varicocele development. However it was showed that there are males without varicocele who have incompetent venous valve system and males with varicocele who had competent venous valves [29].

Second, because the left testicular vein is longer than the right, the hydrostatic pressure difference could be a factor causing a left varicocele. Although the left testicular vein is longer than the right, the simple difference in hydrostatic pressure of a standing column of blood can not be the only reason for development of a varicocele because all males would be affected.

A third theory known as a 'nutcracker effect' is speculated to occur when the testicular vein is compressed between the superior mesenteric artery and aorta. The increase in hydrostatic pressure results in varicocele formation. However although high left renal vein to vena cava pressure gradients are noted in patients with varicocele, it is not a consistent feature [31,32].

More recently it has been hypothesized that increased arterial blood flow to the testis at puberty exceeds the venous capacity resulting in venous dilatation and a varicocele [33,34]. This is consistent with the findings noted in all animal models studied to date, however confirmation in humans will be necessary.

PATHOPHYSIOLOGY

The pathophysiology of varicocele can be studied in animal models by partial ligation of the left renal vein [35]. Many features of the human condition, like increased temperature of the affected testis, increased arterial blood flow and histopathological changes can be replicated in animal models.

The following theories attempt to explain the deleterious effect of varicocele on testicular function.

Hyperthermia

The presence of a varicocele is associated with elevated scrotal and testicular temperature and altered spermatogenesis. Experimental studies have shown that spermatogenesis occurs optimally at temperatures lower than body temperature. Many of the enzymes responsible for optimal DNA synthesis in the testis are temperature dependent [36,37]. The scrotal position of the testis and the cooling system provided by the pampiniform plexus surrounding the testicular artery allows for heat exchange and is responsible for regulating optimal temperature for spermatogenesis [38]. Stasis of blood in the varicocele with resultant increased temperature may be responsible for the deleterious effect of varicocele on spermatogenesis [39]. Increased temperature is associated with decreased number of spermatogonia and increased apoptosis of germinal epithelium cells [40].

Hypoxia and 'adrenal reflux'

Stasis of blood in pampiniform plexus could affect partial oxygen pressure and change aerobic metabolism in the testis. However hypoxia has not been demonstrated in testicular venous blood sampling in humans or experimental animals [41,42]. Reflux of blood down the testicular vein has been demonstrated in patients with varicocele [43,44]. Therefore exposure of the testis to adrenal or renal metabolites is hypothesized as cause for testicular damage. However adrenal or renal metabolites at the level of the testis have not been documented [35,45]. Adrenalectomy done on rats with experimental varicocele did not diminish the effects of the varicocele [46,47]. Thus the adrenal/renal reflux theory does not appear to be responsible for the testicular damage associated with varicocele [46,48].

Abnormal blood flow

A current hypothesis assumes that increased blood flow through the testis can affect spermatogenesis [49,50]. An increase in hydrostatic pressure with a change in filtration pressure could considerably

alter the composition of the interstitial fluid [51]. This alteration conceivably could alter the intimate paracrine communications between the Leydig cells, peritubular myoid cells and Sertoli cells ultimately affecting spermatogenesis [1]. The myoid cells and capillary epithelium undergo pathological changes in association with varicocele that may effect transmembrane transport of substrates to the germinal epithelium [52].

Endocrine imbalance

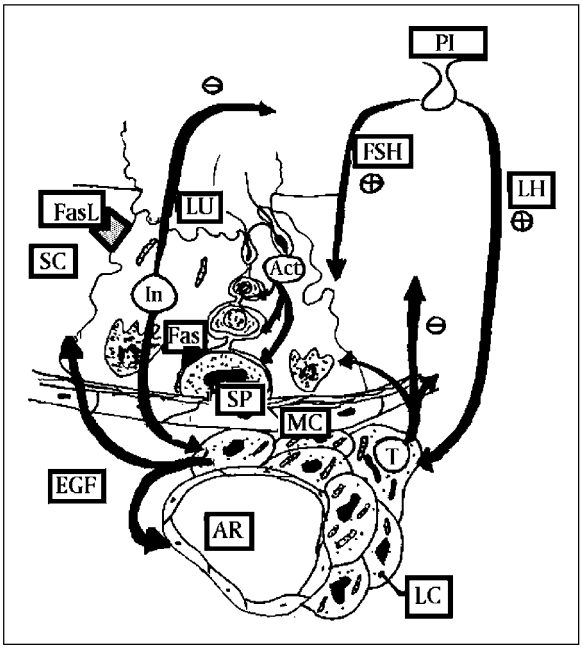
Puberty, spermatogenesis and testicular development are regulated by the hypothalamic-pituitary-testicular axis. There is a wide array of endocrine abnormalities associated with varicocele.

Leydig cells are under the control of luteinizing hormone (LH) and responsible for testosterone production. Some studies have shown that the serum testosterone level may be affected by varicocele, however it is intratesticular testosterone that is important in regulation of spermatogenesis [53,54]. In experimental animal models a varicocele can result in decreased intratesticular testosterone level [55]. The results of human studies are mixed. Ando et al. found reduced serum testosterone level in males with varicocele and increase in serum testosterone level after repair of varicocele [56,60]. Swerdloff and Walsh however showed that there was no difference in testosterone level between males with and without varicocele [61].

Increased LH serum levels and an abnormal response to gonadotropin releasing hormone (GnRH) could implicate a compromise of the hypothalamic-pituitary-gonadal axis involved in the control of testosterone level and spermatogenesis; a pattern similar to hypergonadotropic hypogonadism [62,63]. Increased LH level results in Leydig cells hyperplasia; a known histologic finding in varicocele testicular biopsies [64-66].

Sertoli cell responsiveness to FSH may be diminished in varicocele patients. Stimulation of Sertoli cells with FSH reversed spermatogenesis arrest in experimental animal models [67]. Altered levels of serum inhibin found in patients with varicocele may reflect altered function of Sertoli cells [68]. Cameron et al. noticed that the Sertoli-germ cell junctional complexes appeared to be structurally abnormal in patients with varicocele. They concluded that testicular disruption associated with a varicocele is a phenomenon of the adluminal compartment, and that the Sertoli cell is more sensitive to perturbation of the testicular environment than are germ cells. The Sertoli cell may be the primary intratubular site of alteration leading secondarily to spermatogenic disruption [69]. Histologic studies of the testis from patients with varicocele showed absent germ cells or altered spermatogonia to Sertoli cell ratio – a signs often associated with Sertoli cell dysfunction [70].

Figure 3. Endocrine regulation of spermatogenesis: PI – pituitary gland, hypothalamus, SC – Sertoli cells, LU – lumen of seminiferous tubule, LC – Leydig cells, AR – arteriole, MC – myoid cells, SP – spermatogonia, T – testosterone, FSH – follicle stimulating hormone, LH – luteinizing hormone, In – inhibin, Act – actvin, EGF – epidermal growth factor, FasL and Fas – ligand and receptor system involved in apoptosis of germ cells, (–) decrease (negative feed back), (+) increase (positive feed back). Sertoli cells (SC) under the influence of FSH regulate spermatogenesis by activin (Act), androgen binding protein and direct interactions with spermatogonia and spermatids. Leydig cells regulate spermatogenesis by achieving high intratesticular testosterone concentration. Epidermal growth factor (EGF) is produced by LC and controls mitotic divisions of germinal epithelium. EGF also stimulates divisions of peritubular myoid cells which in return produce another paracrine hormone peritubular myoid substance (PmodS).



Paracrine regulations of the testis.

Insight into the detailed mechanism of spermatogenesis is even more complicated as spermatogenesis

is also regulated by complex interactions and signals at the cellular level in the testis [1] (Figure 3).

Both Sertoli cells (SC) and Leydig cells (LC) regulate spermatogenesis by steroidogenesis and growth factors production [71,72].

Sertoli cells, tightly regulate germ cell proliferation and differentiation and are implicated in the control of germ cell apoptosis. Fas (APO-1, CD95), a transmembrane receptor protein expressed by germ cells, transmits an apoptotic signal within cells when bound by Fas ligand (FasL) produced by Sertoli cells. The Fas system has been implicated in immune regulation, including cytotoxic T cell-mediated cytotoxicity, activation-induced suicide of T cells, and control of immune-privileged sites [73]. SC stimulated by FSH produce inhibin (In) and activin (Ac) [74]. Inhibin has negative feedback control on pituitary and FSH secretion. Inhibin also binds to Leydig cells (LC) regulating testosterone (T) production. Activin binds to round spermatids and spermatogonia (SP), effecting spermatogenesis. Spermatogonia are known to stimulate transferrin production by SC by an unidentified protein substance [75].

Leydig cells control spermatogensis not only by steroids production but also by epidermal growth factors (EGF) which binds to spermatogonia and spermatids regulating cell divisions [76]. Receptors for transforming growth factor (TGF), one of the EGFs produced by LC, were found in peritubular myoid cells [77]. Peritubular myoid cells (PC) secrete peritubular myoid cell substance (PmodS) which stimulates SC. LC control adluminal tubular compartment and transport of nutrients from the vascular space to germinal epithelium by vascular endothelial growth factor (VEGF). VEGF is of particular interest in varicocele since VEGF regulates endothelial permeability and is a angioproliferative factor [78].

Locally produced neurotropins play their distinct role in spermatogenesis regulation [79]. Opioids receptors are found on LC. During stress, release of endorphins stimulate opioid receptors and decreases testosterone production. Blocking the opioid receptors by naloxone restores testosterone production to normal [80].

With each discovery of a new paracrine substance, and a better understanding of molecular mechanisms controlling spermatogenesis we come closer to the time when we will accurately predict which adolescent will require surgical or medical interventions for testicular dysfunction.

PATHOLOGY

Testicular hypotrophy

Testicular function most effected by the varicocele is spermatogenesis. 81The most common findings on semen analysis are: increased number of pathological sperm forms, decreased motility and decreased sperm density [8,82,83]. Sperm analysis in adolescents with varicocele shows decreased sperm density, increased number of pathological forms and decreased motility however there are no established norms for adolescent semen analysis [82]. Varicocele is also associated with testicular growth arrest in adolescents [10,12,84]. Testicular growth arrest may be considered the hallmark of testicular damage in adolescent varicocele. Significant volume loss in adolescents with varicocele has been noted in 77% of boys, 10% of whom had a left testis one-fourth the size of the right testis. 85 Testicular hypotrophy is time dependent [86,87].

Testicular volume during preadolescents is constant and at the onset of puberty the testis suddenly increases in size even prior to other pubertal changes. In adolescents with a varicocele the rapid growth of the testis between the ages of 11 and 16 is effected by the varicocele and results in a volume discrepancy between the right and left testis.

Histopathology

Testicular biopsy in males with varicocele shows a wide array of abnormalities. The most common findings are Leydig cells hyperplasia, decreased number of spermatogonia per tubule, spermatogenesis arrest and sloughing of germinal epithelium [88-92]. A thickened basement membrane of seminiferous tubules and proliferative lesions of endoepithelium are often demonstrated and may affect transportation of oxygen and glucose through these structures [93,94].

DIAGNOSIS

Since the adolescent with a varicocele is often asymptomatic it is usually found on routine physical exam. The patient should be examined standing in a warm room to relax the scrotum and allow easier examination of the spermatic cord. The scrotum is first visually inspected for any obvious dis-

tention around the spermatic cord; a visible varicocele is considered a large or Grade 3 varicocele. The scrotum, testes, and cord structures are then gently palpated. A palpable varicocele has been described as feeling like a bag of worms or a squishy tube. More subtle varicoceles may feel like a thickened or asymmetric cord. The nonvisible, but palpable varicocele, is considered to be moderate in size (Grade 2). If a varicocele is not palpable but the patient performs a Valsalva maneuver which distends the pampiniform plexus of veins, then a small (Grade 1) varicocele is present. After examining the patient in the standing position, the patient should be examined supine. A thickened cord due to a varicocele should resolve in the supine position, whereas a thickened cord due to a lipoma will not change when the patient is supine. Secondary varicocele especially on the right side should always be excluded since it can be caused by serious conditions like retroperitoneal tumors, kidney tumors or lymphadenopathy [95]. Idiopathic varicocele is more prominent in the upright position and disappears in the supine position. A secondary varicocele does not change its size so dramatically in the supine position.

Testicular size needs to be measured to determine if the varicocele is adversely affecting the growth of the testis. The volume of a normal testis measures 1 to 2 ml in the prepubertal male. Due to extensive individual variation in normal growth and development, testicular size is correlated with Tanner Stage, growth velocity, and bone age rather than chronological age. 1

A number of methods have been used to measure the size of the testis. These include visual comparison, rulers, calipers, comparative ovoids (Prader Orchidometer), punched-out elliptical rings (Takahara Orchidometer), and ultrasound. A high correlation ($r = 0.992$) between ultrasound and actual volume was noted and was shown to be highly reproducible [96]. The Prader Orchidometer was shown to correlate with ultrasound measurement in 256 patients ($r = 0.91$), though the degree of correlation was dependent upon the investigator's clinical experience. In a clinical study of 22 male adolescents with a varicocele, 24% of patients with growth arrest would have been missed if measured by Prader Orchidometer alone, and three patients felt to have a significant size discrepancy (>2 ml) by Prader Orchidometer measurements were found to be normal by ultrasound volume estimate. These findings indicate that clinical estimates of testicular size by the Prader Orchidometer are not as accurate or reproducible as those determined by ultrasound. Accurate measurement is important because operative decisions rest in the balance [97].

There is significant disagreement as to what constitutes a significant size discrepancy justifying surgical intervention. Testicular ultrasound is the most accurate and reproducible method to assess testicular volume and significant testicular size variations. A volume difference of less than 2 ml can be due to the measurement technique alone. Therefore, size variation of greater than 2 ml by ultrasound is currently the best indicator of testicular damage and should serve as the minimal requirement for surgical repair of the adolescent varicocele [1]. Surgical intervention reverses testicular growth arrest and assessment of testicular volume postoperatively predicts resolution of the varicocele [12,98].

MANAGEMENT

There are some cardinal questions to be answered regarding management of adolescent varicocele.

Is it justified to promote the awareness about varicocele among pediatricians and primary care providers and to look for varicocele in asymptomatic adolescents?

There is sufficient evidence that varicocele is associated with testicular growth arrest in adolescents and varicocelectomy results in testis 'catch-up' growth [8-10]. Lenzi et al. showed that early varicocele repair in adolescents resulted in better semen analysis results than in untreated adolescents after 2-8 years of follow up [13]. Based on these studies it seems justified to encourage non-urologists to look for varicocele in the adolescents during physical examination. Examination of the genitalia at puberty also allows the clinician to find other urologic abnormalities like cryptorchidism, hernia, curvature of penis and improve the health of adolescents [99,102].

Once a varicocele has been found, what information needs to be given to the patient and his parents?

A number of psychological reactions (anxiety, depressed mood) were experienced in approximately 30% of boys who were informed about varicocele [103]. Since the word 'infertility' is often associated with sexual impairment we believe that

during discussion with the patient and his parents the only fact which should be stressed is that the varicocele may result in a decrease in testicular volume that can be reversed by surgical treatment. However it is hard to discount the association between varicocele and infertility in this era of common accessibility to the medical literature on the Internet (we found 10 World Wide Web pages searching for the term 'varicocele', all had the word 'infertility' in the text). Since there are studies which demonstrate an abnormal semen analysis in adolescents it seems advisable to discuss all the findings first with the parents who can be helpful in presentation of the problem to the child [104,105].

Once diagnosed; who should we treat?

Varicocele is the most common correctable cause of male infertility [106]. A recent metaanalysis of the literature done by Pryor and Howards showed that two thirds of patients will have improvement in semen analysis after varicocele repair, and 40% of partners will become pregnant [16]. Historically adolescent varicocele was left untreated since its relation to infertility was not well established. Subsequently Kass and Belman showed that testicular growth arrest could be reversed by varicocelectomy in adolescents [107]. Repair of varicocele reverses not only the growth arrest but also improved semen analysis in adolescents and young males [105,108,109].

There is good evidence that with time, if left untreated, the varicocele will continue to effect testicular growth with progressive loss of volume and progressive deterioration in semen analysis [6,84]. Goldstein suggests that varicocele causes a progressive decline in fertility and that prior fertility in men with varicocele does not predict resistance to varicocele induced impairment of spermatogenesis in the furture so called 'secondary infertility' [6].

The association between varicocele and infertility is well established [8]. The most difficult question is which clinical test should we use to establish the indications for surgical treatment in an adolescent with a varicocele.

Currently the clinical tests used to establish indications for varicocele repair are:

1. Grade of varicocele
2. Measurement of testicular volume to assess testicular growth arrest.
3. Gonadotropin releasing hormon (GnRH) stimulation test
4. Measurement of pampiniform veins diameter
5. Serum luteinizing hormone (LH), follicle-stimulating hormone (FSH) and inhibin levels

Varicocele grade

Varicocele grade does not correlate well with abnormal spermiograms or infertility in adults [110]. There are different opinions regarding correlation between grade of varicocele and degree of testicular hypotrophy in adolescents. Lyon and associates found no correlation of varicocele grade and testicular size in 30 adolescents [85]. In contrast Skoog, Steeno, and Paduch all independently noticed that boys with severe varicocele have a smaller ipsilateral testicle [10,97,103]. It was also noticed that the smaller the testis the worse the semen analysis results [82,8]. However grade of varicocele by itself should not be the sole indication for treatment.

Testicular volume

There is an abundance of literature confirming that varicocele is associate with testicular growth arrest in adolescents and varicocele repair results in testicular 'catch-up' growth [10,105,108,109]. Testicular growth arrest with volume difference of more than 2 ml assessed by ultrasonography is the most common indication for treatment [10]. The development of secondary infertility is another strong argument for early varicocele repair as if let untreated the varicocele will not only effect testicular volume but also effects spermatogenesis [6]. A decrease in testicular volume is the best indicator for surgical correction of a varicocele. However not every boy with a varicocele and testicular growth arrest will be infertile and there is still a need to search for a test which would better distinguish between adolescents with varicocele who will develop infertility and those who will remain fertile. In adult males the situation is a little simpler because the indications for surgery are usually established after 12 months of infertility confirmed by abnormal semen analysis and the presence of a varicocele. Obtaining a semen sample in adolescents is possible but difficult.

Gonadotropin releasing hormone (GnRH) stimulation test

Damage to germinal epithelium results in compensatory stimulation of the pituitary gland and subse-

quent increase in FSH and LH production by gonadotrophs [111]. Intravenous administration of GnRH stimulates the pituitary gland to release FSH and LH. FSH levels are elevated in any condition (like varicocele) effecting the integrity of germinal epithelium [112]. In theory GnRH stimulation test could distinguish between adolescents with a varicocele who have abnormal testicular functions and those who have normal spermatogenesis [11]. However clinical practice showed that the GnRH stimulating test is expensive, requires multiple serum samples and lacks the association between abnormal results, growth arrest and infertility [113,114]. An abnormal GnRH stimulating test was found in 30% of adolescents with varicocele and was not correlated with atrophy or infertility [115]. Currently it seems that GnRH stimulation test has limited role in clinical evaluation of adolescent with a varicocele.

Pampiniform plexus veins diameter

Ultrasonographic measurement of pampiniform plexus veins diameter (PPVD) has been used to look for subclinical varicocele in adult infertile population when physical exam is inconclusive and to follow persistent varicocele but PPVD measurements are not useful in adolescents [116,117].

Inhibin level

The serum inhibin levels reflect the integrity of the seminiferous tubule. However there is not enough data to use serum inhibin levels in clinical decisions at this time [68,74,118-120].

Currently, prophylactic surgery for every adolescent with varicocele is not advised since it would result in treatment of 15% of adolescents.

In summary it seems that treatment should be offered to:

1. adolescents with testicular growth arrest (2 SD from normal testicular growth curves, more than 2 cc of difference between left and right testicle)
2. adolescents with abnormal semen analysis with high-grade varicocele
3. adolescents with symptoms: pain, heaviness, swelling
4. adolescents with bilateral varicoceles

TREATMENT OPTIONS

Treatment options in the management of adolescent with a varicocele originated from the practice of adult male infertility and subsequently were applied to the treatment of adolescents.

Varicocele repair can be done by surgical ligation and division of testicular veins or intravenous embolization of testicular veins.

Three open surgical approaches are currently used: subinguinal approach (Marmar), inguinal approach (Ivanissevich) and retroperitoneal approach (Palomo). Laparoscopic varicocele ligation has often been used in adults. Embolization techniques, regardless of embolizing material, can be divided into: antegrade (infusion through scrotal part of pampiniform plexus veins) and retrograde (catheter placed through femoral vein puncture) infusion.

The failure rate, frequency of complications, cost and outcome are important factors which need to be evaluated in choosing preferable treatment option in adolescent repair (Table 2). It is important to remember that the majority of studies on varicocele repair relates to adult infertile males with varicocele and not adolescents.

Failure rate

Failure of treatment is defined here as a persistent or (rarely) recurrence of the varicocele after the

Table 2. Surgical approach, complications and relative costs of varicocele repair.

Technique	Hydrocele rate	Failure rate	Cost	References
Retroperitoneal	7-10%	9-11% (artery sparing) <3% (artery taking)	Low	121 10 12
Inguinal	3-7%	9-12%	Low	123 129 147
Inguinal microscopic	< 1%	2.1% 0.6%	Moderate	124 125
Laparoscopic	1.25%	9% 1.25% in adolescents	High	148 149
Embolization		19%	High	135

repair and can occur in 9% to 16% of adolescents [121]. Persistence of the varicocele results in lack of 'catch-up' testicular growth [12]. Most authors attribute the high recurrence rate to missed venous collaterals which run parallel to the main testicular vein. The collaterals can be quite difficult to identify and ligate separately from the testicular artery. Reported persistence rate using artery sparring retroperitoneal approach ranges from 3% to 11% [12,121-123]. Ligation of both testicular vein trunci and the artery has the advantage of a decreased persistence rate and does not result in testicular atrophy since the testis has collateral arterial blood supply from the cremasteric and deferential artery [12,21]. Atassi and colleagues achieved a persistence rate below 2% in adolescents treated by high retroperitoneal ligation with testicular artery ligation [12].

There is, however, some objection to simultaneous ligation of the testicular artery in men with previous inguinal surgery since there may be compromised blood supply from the cremasteric and deferential arteries. Interruption of the testicular artery in these patients has a high probability of developing testicular atrophy. Also, subsequent vasectomy in patients with testicular artery division should be avoided since ligation of the vasal artery could result in testicular atrophy in the absence of the testicular artery.

The high persistence rate and postoperative hydrocele rate resulted in the development of microsurgical inguinal approaches for correction of the varicocele. Both subinguinal and inguinal microsurgical repair are used quite often in adults with varicocele and indeed offer lower persistence rates and a low incidence of postoperative hydrocele [124-126]. The low persistence rate using the microsurgical inguinal repair is attributed to ligation of all distended vein trunci and collaterals at the level of the deep inguinal ring. [124-126]. Experience in microsurgical inguinal varicocele repair in adolescent is limited. Reports by Minevitch and Goldstein demonstrated a significantly lower rate of persistence and postoperative hydrocele. [127,128]. The microscopic approach allows one to ligate only the veins leaving the lymphatic vessels intact what decreased the postoperative hydrocele rate to less than 1% [125,126].

Laparoscopic varicocele repair with, and without artery sparing modifications seems to be suitable treatment technique especially since recent reports in adults showed a lower rate of persistence [129-131]. Laparoscopic surgery in the pediatric population is gaining acceptance but it bares the risk of significant complications like bowel perforation, major vascular injury, pneumothorax, and incisional hernia. There is not much experience with laparoscopic varicocele ligation in the adolescent population [132-134].

Retrograde embolization, unfortunately is associated with an unacceptable high rate of persistence and is the most expensive of treatment techniques [1]. Possible explanation of such a high persistence rate of the varicocele after embolization can be attributed to the highly variable anatomy of the testicular venous drainage and technical difficulties [135]. Antegrade embolization is more often used to treat persistent varicoceles than as an initial treatment [136-139].

Other options to decrease the rate of varicocele persistence are intraoperative venography and methylene blue injections [27,123,140,141]. Intraoperative venography in theory should facilitate ligation of all testicular vein trunci and decrease the rate of persistence. Hart recommends routine use of intraoperative venography because 16% of their 62 patients had missed venous vessels after initial venous ligation [145]. Similar conclusions, based on a decreased persistence rate, were also made by Levitt, Zaontz and Gill [143,145]. However Palmer and Kass reported no difference in their rate of varicocele persistence after repair with and without intraoperative venography [121,123,141]. Based on these studies intraoperative venography offer little benefit in the repair of adolescents with a varicocele.

Based on our review of the literature and the results of a survey of pediatric urologists in the United States high retroperitoneal ligation of the testicular artery and veins offers the best results in adolescents. Although currently the high retroperitoneal approach is a treatment of choice in adolescent varicocele, pediatric urologists should consider using the microscopic inguinal or subinguinal approach with arterial preservation.

CONCLUSIONS

The adolescent with a varicocele presents the clinician with an interesting and challenging problem. There is a great need for further basic research to help better select the patient who needs surgical correction of his varicocele.

We have outlined recommendations which can be used in everyday practice. Each clinician needs to make his/her own decisions regarding who, when and how to treat the adolescent with varicocele.

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Articles
TheScientificWorldJOURNAL (2004) 4 (S1), 263–278
ISSN 1537-744X; DOI 10.1100/tsw.2004.76

Diagnosis, Evaluation and Treatment of Adolescent Varicocele

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Previously published in the Digital Urology Journal

DOMAIN: urology

INTRODUCTION

A varicocele can be defined as an abnormal tortuosity and dilation of the veins of the pampiniform plexus. Idiopathic varicocele is usually asymptomatic. It is noticed as an asymmetry in scrotal size, and presents as heaviness in the scrotum, or rarely with testicular pain. In most cases the adolescent is unaware of the varicocele and it is discovered during a regular physical examination or during examination for military service.[1-4]

The incidence of high-grade varicocele is approximately 5 % throughout the world.[5] Varicocele is associated with a time dependant growth arrest in adolescents and adult males.[6] There is a clear association between varicocele, infertility and testicular growth arrest.[7-9] It is also known that varicocelectomy can reverse testicular growth arrest in adolescents.[10-13] These facts have raised the question of how to best manage the adolescents with a varicocele.

Adolescents do not present with infertility and thus should prophylactic repair be performed to prevent infertility in the future? Who would benefit the most by varicocelectomy: the adolescents with testicular growth arrest or any adolescent with a varicocele? Is it better to wait for a semen analysis or offer earlier treatment based on testicular growth arrests? These questions can be only answered when we have better understanding of the pathophysiology of varicocele.

The purpose of this review is to present the current literature on adolescent varicocele and provide guidelines to the clinician how to manage the adolescents with a varicocele.

EPIDEMIOLOGY

In the general population of healthy males the overall incidence of varicocele (all grades) is 10% to 15%.[4,5,14,15] Approximately 30-50 % of males with primary infertility have a varicocele.[16-19] Varicocele is most common on the left side. Varicocele appears at early puberty however it can occasionally be found in preadolescent boys.[2,20] The incidence in older adolescence varies from 12.4 % to 17.8 % with an average of 14.7 %, similar to the incidence in adult males. (Table 1.)

TABLE 1
Incidence of varicocele in general population of healthy adolescents.

Reference:	No of patients:	Age:	Incidence: (total)
Oster, 1971[101]	837	10-19	16.2%
Steeno, 1976 [103]	4067	12-25	14.7%
Yerokhin, 1979 [146]	10000	10-17	12.4%
Belloli, 1993 [9]	9861	10-16	16.0%
Niedzielski, 1997 [5]	2478	10-20	17.8%

ANATOMY

The arterial blood supply to the testis comes from the testicular artery, vasal artery and the cremasteric artery. At the level of testis all three arteries anastomose to allow adequate blood supply even with division of the testicular artery.[21,22]

The venous drainage (Figure 1) is more complicated with many individual variations. Above the testis is a network of communicated veins called the pampiniform plexus (PP), the drainage from PP is via the testicular vein trunci, pudendal veins and cremasteric veins.[23,24] In most cases the testicular vein trunci form a single testicular vein entering the renal vein on the left and the inferior vena cava on the right. Venographic studies have demonstrated that the left testicular vein can rarely enter the inferior vena cava, and there are communications between the testicular vein and the inferior vena cava below the level of the renal veins.[25-27]

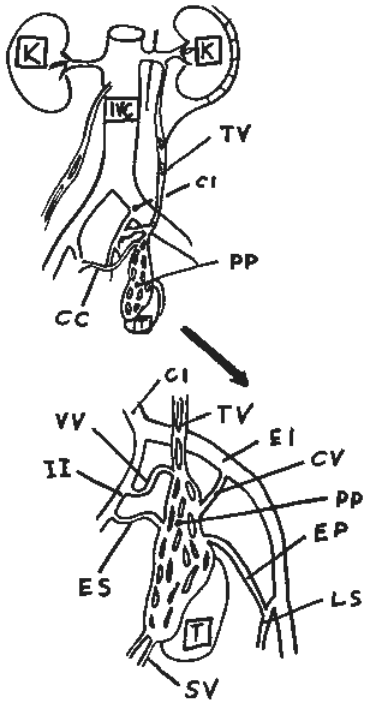

FIGURE 1. Anatomy of venous drainage from left testis.

There are also cross-communications between the left and right testicular venous systems (Figure 2).[26,28-30]

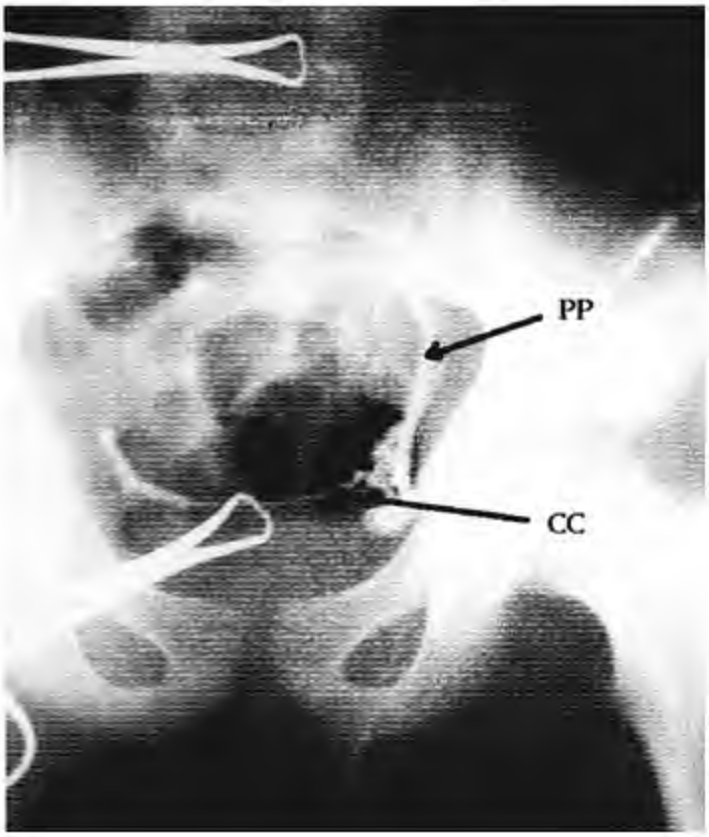

FIGURE 2. Intraoperative venogram showing left to right cross-communicating veins CC-cross-communications, PP-pampiniform plexus veins

ETIOLOGY

There are several theories attempting to explain the etiology of varicocele. The predominance of the left side varicocele and the unique anatomy of the left testicular vein are the basis for several theories explaining the etiology of varicocele.

The presence of venous valves was long believed to be a guarding mechanism against developing a varicocele and incompetence of the venous valve system was thought to be responsible for varicocele development. However, it was shown that there are males without varicocele who have incompetent venous valve system and males with varicocele who had competent venous valves.[29]

Second, because the left testicular vein is longer than the right, the hydrostatic pressure difference could be a factor causing a left varicocele. Although the left testicular vein is longer than the right, the simple difference in hydrostatic pressure of a standing column of blood can not be the only reason for development of a varicocele because all males would be affected.

A third theory known as a "nutcracker effect" is speculated to occur when the testicular vein is compressed between the superior mesenteric artery and aorta. The increase in hydrostatic pressure results in varicocele formation. However although high left renal vein to vena cava pressure gradients are noted in patients with varicocele, it is not a consistent feature.[31,32]

More recently it has been hypothesized that increased arterial blood flow to the testis at puberty exceeds the venous capacity resulting in venous dilatation and a varicocele.[33,34] This is consistent with the findings noted in all animal models studied to date, however confirmation in humans will be necessary.

PATHOPHYSIOLOGY

The pathophysiology of varicocele can be studied in animal models by partial ligation of the left renal vein.[35] Many features of the human condition, like increased temperature of the affected testis, increased arterial blood flow and histopathological changes can be replicated in animal models.

The following theories attempt to explain the deleterious effect of varicocele on testicular function.

Hyperthermia

The presence of a varicocele is associated with elevated scrotal and testicular temperature and altered spermatogenesis. Experimental studies have shown that spermatogenesis occurs optimally at temperatures lower than body temperature. Many of the enzymes responsible for optimal DNA synthesis in the testis are temperature dependent.[36,37] The scrotal position of the testis and the cooling system provided by the pampiniform plexus surrounding the testicular artery allows for heat exchange and is responsible for regulating optimal temperature for spermatogenesis.[38] Stasis of blood in the varicocele with resultant increased temperature may be responsible for the deleterious effect of varicocele on spermatogenesis.[39] Increased temperature is associated with decreased number of spermatogonia and increased apoptosis of germinal epithelium cells.[40]

Hypoxia and "adrenal reflux"

Stasis of blood in pampiniform plexus could affect partial oxygen pressure and change aerobic metabolism in the testis. However hypoxia has not been demonstrated in testicular venous blood sampling in humans or experimental animals.[41,42] Reflux of blood down the testicular vein has been demonstrated in patients with varicocele.[43,44] Therefore exposure of the testis to adrenal or renal metabolites is hypothesized as cause for testicular damage. However adrenal or renal metabolites at the level of the testis have not been documented.[35,45] Adrenalectomy done on rats with experimental varicocele did not diminish the effects of the varicocele.[46,47] Thus the adrenal/renal reflux theory does not appear to be responsible for the testicular damage associated with varicocele.[46,48]

Abnormal blood flow

A current hypothesis assumes that increased blood flow through the testis can affect spermatogenesis.[49,50] An increase in hydrostatic pressure with a change in filtration pressure could considerably alter the composition of the interstitial fluid.[51] This alteration conceivably could alter the intimate paracrine communications between the Leydig cells, peritubular myoid cells and Sertoli cells ultimately affecting spermatogenesis.[1] The myoid cells and capillary epithelium undergo pathological changes in association with varicocele that may effect transmembrane transport of substrates to the germinal epithelium.[52]

Endocrine imbalance

Puberty, spermatogenesis and testicular development are regulated by the hypothalamic-pituitary-testicular axis. There is a wide array of endocrine abnormalities associated with varicocele.

Leydig cells are under the control of luteinizing hormone (LH) and responsible for testosterone production. Some studies have shown that the serum testosterone level may be affected by varicocele, however it is intratesticular testosterone that is important in regulation of spermatogenesis.[53,54] In experimental animal models a varicocele can result in decreased intratesticular testosterone level.[55] The results of human studies are mixed. Ando et al. found reduced serum testosterone level in males with varicocele and increase in serum testosterone level after repair of varicocele.[56-60] Swerdloff and Walsh however showed that there was no difference in testosterone level between males with and without varicocele.[61]

Increased LH serum levels and an abnormal response to gonadotropin releasing hormone (GnRH) could implicate a compromise of the hypothalamic-pituitary-gonadal axis involved in the control of testosterone level and spermatogenesis; a pattern similar to hypergonadotropic hypogonadism.[62,63] Increased LH level results in Leydig cells hyperplasia; a known histologic finding in varicocele testicular biopsies.[64-66]

Sertoli cell responsiveness to FSH may be diminished in varicocele patients. Stimulation of Sertoli cells with FSH reversed spermatogenesis arrest in experimental animal models.[67] Altered levels of serum inhibin found in patients with varicocele may reflect altered function of Sertoli cells.[68] Cameron et al. noticed that the Sertoli-germ cell junctional complexes appeared to be structurally abnormal in patients with varicocele. They concluded that testicular disruption associated with a varicocele is a phenomenon of the adluminal compartment, and that the Sertoli cell is more sensitive to perturbation of the testicular environment than are germ cells. The Sertoli cell may be the primary intratubular site of alteration leading secondarily to spermatogenic disruption.[69] Histologic studies of the testis from patients with varicocele showed absent germ cells or altered spermatogonia to Sertoli cell ratio.[70]

Paracrine regulations of the testis

Insight into the detailed mechanism of spermatogenesis is even more complicated as spermatogenesis is also regulated by complex interactions and signals at the cellular level in the testis.[1] (Figure 3)

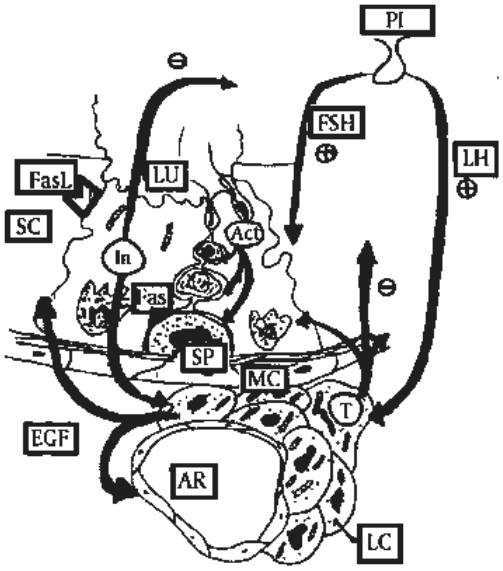

FIGURE 3. Endocrine regulation of spermatogenesis.

Both Sertoli cells (SC) and Leydig cells (LC) regulate spermatogenesis by steroidogenesis and growth factors production.[71,72]

Sertoli cells, tightly regulate germ cell proliferation and differentiation and are implicated in the control of germ cell apoptosis. Fas (APO-1, CD95), a transmembrane receptor protein expressed by germ cells, transmits an apoptotic signal within cells when bound by Fas ligand (FasL) produced by Sertoli cells. The Fas system has been implicated in immune regulation, including cytotoxic T cell-mediated cytotoxicity, activation-induced suicide of T cells, and control of immune-privileged sites.[73] SC stimulated by FSH produce inhibin (In) and activin (Ac).[74] Inhibin has negative feedback control on pituitary and FSH secretion. Inhibin also binds to Leydig cells (LC) regulating testosterone (T) production. Activin binds to round spermatids and spermatogonia (SP), effecting spermatogenesis. Spermatogonia are known to stimulate transferrin production by SC by an unidentified protein substance.[75]

Leydig cells control spermatogensis not only by steroids production but also by epidermal growth factors (EGF) which binds to spermatogonia and spermatids regulating cell divisions.[76] Receptors for transforming growth factor (TGF), one of the EGFs produced by LC, were found in peritubular myoid cells.[77] Peritubular myoid cells (PC) secrete peritubular myoid cell substance (PmodS) which stimulates SC. LC control adluminal tubular compartment and transport of nutrients from the vascular space to germinal epithelium by vascular endothelial growth factor (VEGF). VEGF is of particular interest in varicocele since VEGF regulates endothelial permeability and is a angioproliferative factor.[78]

Locally produced neurotropins play their distinct role in spermatogenesis regulation.[79] Opioids receptors are found on LC. During stress, release of endorphins stimulate opioid receptors and decreases testosterone production. Blocking the opioid receptors by naloxone restores testosterone production to normal.[80]

With each discovery of a new paracrine substance, and a better understanding of molecular mechanisms controlling spermatogenesis we come closer to the time when we will accurately predict which adolescent will require surgical or medical interventions for testicular dysfunction.

PATHOLOGY

Testicular hypotrophy

Testicular function most effected by the varicocele is spermatogenesis.[81] The most common findings on semen analysis are: increased number of pathological sperm forms, decreased motility and decreased sperm density.[8,82,83] Sperm analysis in adolescents with varicocele shows decreased sperm density, increased number of pathological forms and decreased motility however there are no established norms for adolescent semen analysis.[82] Varicocele is also associated with testicular growth arrest in adolescents.[10,12,84] Testicular growth arrest may be considered the hallmark of testicular damage in adolescent varicocele. Significant volume loss in adolescents with varicocele has been noted in 77 % of boys, 10% of whom had a left testis one-fourth the size of the right testis.[85] Testicular hypotrophy is time dependent.[86,87]

Testicular volume during preadolescents is constant and at the onset of puberty the testis suddenly increases in size even prior to other pubertal changes. In adolescents with a varicocele the rapid growth of the testis between the ages of 11 and 16 is effected by the varicocele and results in a volume discrepancy between the right and left testis.

HISTOPATHOLOGY

Testicular biopsy in males with varicocele shows a wide array of abnormalities. The most common findings are Leydig cells hyperplasia, decreased number of spermatogonia per tubule, spermatogenesis arrest and sloughing of germinal epithelium.[88-92] A thickened basement membrane of seminiferous tubules

and proliferative lesions of endoepithelium are often demonstrated and may affect transportation of oxygen and glucose through these structures.[93,94]

DIAGNOSIS

Since the adolescent with a varicocele is often asymptomatic it is usually found on routine physical exam. The patient should be examined standing in a warm room to relax the scrotum and allow easier examination of the spermatic cord. The scrotum is first visually inspected for any obvious distention around the spermatic cord; a visible varicocele is considered a large or Grade 3 varicocele. The scrotum, testes, and cord structures are then gently palpated. A palpable varicocele has been described as feeling like a bag of worms or a squishy tube. More subtle varicoceles may feel like a thickened or asymmetric cord. The nonvisible, but palpable varicocele, is considered to be moderate in size (Grade 2). If a varicocele is not palpable but the patient performs a Valsalva maneuver which distends the pampiniform plexus of veins, then a small (Grade 1) varicocele is present. After examining the patient in the standing position, the patient should be examined supine. A thickened cord due to a varicocele should resolve in the supine position, whereas a thickened cord due to a lipoma will not change when the patient is supine. Secondary varicocele especially on the right side should always be excluded since it can be caused by serious conditions like retroperitoneal tumors, kidney tumors or lymphadenopathy.[95] Idiopathic varicocele is more prominent in the upright position and disappears in the supine position. A secondary varicocele does not change its size so dramatically in the supine position.

Testicular size needs to be measured to determine if the varicocele is adversely affecting the growth of the testis. The volume of a normal testis measures 1 to 2 ml in the prepubertal male. Due to extensive individual variation in normal growth and development, testicular size is correlated with Tanner Stage, growth velocity, and bone age rather than chronological age.[1]

A number of methods have been used to measure the size of the testis. These include visual comparison, rulers, calipers, comparative ovoids (Prader Orchidometer), punched-out elliptical rings (Takahara Orchidometer), and ultrasound. A high correlation (r = .992) between ultrasound and actual volume was noted and was shown to be highly reproducible. [96] The Prader Orchidometer was shown to correlate with ultrasound measurement in 256 patients (r = .91), though the degree of correlation was dependent upon the investigator's clinical experience. In a clinical study of 22 male adolescents with a varicocele, 24% of patients with growth arrest would have been missed if measured by Prader Orchidometer alone, and three patients felt to have a significant size discrepancy (>2 ml) by Prader Orchidometer measurements were found to be normal by ultrasound volume estimate. These findings indicate that clinical estimates of testicular size by the Prader Orchidometer are not as accurate or reproducible as those determined by ultrasound. Accurate measurement is important because operative decisions rest in the balance.[97]

There is significant disagreement as to what constitutes a significant size discrepancy justifying surgical intervention. Testicular ultrasound is the most accurate and reproducible method to assess testicular volume and significant testicular size variations. A volume difference of less than 2 ml can be due to the measurement technique alone. Therefore, size variation of greater than 2 ml by ultrasound is currently the best indicator of testicular damage and should serve as the minimal requirement for surgical repair of the adolescent varicocele. [1] Surgical intervention reverses testicular growth arrest and assessment of testicular volume postoperatively predicts resolution of the varicocele.[98]

MANAGEMENT

There are some cardinal questions to be answered regarding management of adolescent varicocele.

Is it justified to promote the awareness about varicocele among pediatricians and primary care providers and to look for varicocele in asymptomatic adolescents?

There is sufficient evidence that varicocele is associated with testicular growth arrest in adolescents and varicocelectomy results in testis "catch-up" growth.[8,10] Lenzi et al. showed that early varicocele repair in adolescents resulted in better semen analysis results than in untreated adolescents after 2-8 years of follow up.[13] Based on these studies it seems justified to encourage non-urologists to look for varicocele in the adolescents during physical examination. Examination of the genitalia at puberty also allows the clinician to find other urologic abnormalities like cryptorchidism, hernia, curvature of penis and improve the health of adolescents.[99-102]

Once a varicocele has been found, what information needs to be given to the patient and his parents?

A number of psychological reactions (anxiety, depressed mood) were experienced in approximately 30% of boys who were informed about varicocele.[103] Since the word "infertility" is often associated with sexual impairment we believe that during discussion with the patient and his parents the only fact which should be stressed is that the varicocele may result in a decrease in testicular volume that can be reversed by surgical treatment. However it is hard to discount the association between varicocele and infertility in this era of common accessibility to the medical literature on the Internet (we found more than 50 World Wide Web pages searching for the term "varicocele", all had the word "infertility" in the text). Since there are studies which demonstrate an abnormal semen analysis in adolescents it seems advisable to discuss all the findings first with the parents who can be helpful in presentation of the problem to the child.[104,105]

Once diagnosed, who should we treat? Varicocele is the most common correctable cause of male infertility.[106] A recent metaanalysis of the literature done by Pryor and Howards showed that two thirds of patients will have improvement in semen analysis after varicocele repair, and 40 % of partners will become pregnant.[16] Historically adolescent varicocele was left untreated since its relation to infertility was not well established. Subsequently Kass and Belman showed that testicular growth arrest could be reversed by varicocelectomy in adolescents.[107] Repair of varicocele reverses not only the growth arrest but also improved semen analysis in adolescents and young males.[105,108,109]

There is good evidence that with time, if left untreated, the varicocele will continue to effect testicular growth with progressive loss of volume and progressive deterioration in semen analysis. [6, 84] Goldstein suggests that varicocele causes a progressive decline in fertility and that prior fertility in men with varicocele does not predict resistance to varicocele induced impairment of spermatogenesis in the furture so called "secondary infertility".[6]

The association between varicocele and infertility is well established.[8] The most difficult question is which clinical test should we use to establish the indications for surgical treatment in an adolescent with a varicocele.

Currently the clinical tests used to establish indications for varicocele repair are:

- Grade of varicocele
- Measurement of testicular volume to assess testicular growth arrest.
- Gonadotropin releasing hormon (GnRH) stimulation test
- Measurement of pampiniform veins diameter
- Serum luteinizing hormone (LH), follicle-stimulating hormone (FSH) and inhibin levels

Varicocele grade

Varicocele grade does not correlate well with abnormal spermiograms or infertility in adults.[110] There are different opinions regarding correlation between grade of varicocele and degree of testicular hypotrophy in adolescents. Lyon and associates found no correlation of varicocele grade and testicular size in 30 adolescents.[85] In contrast Skoog, Steeno, and Paduch all independently noticed that boys with severe varicocele have a smaller ipsilateral testicle.[10,97,103] It was also noticed that the smaller the testis the worse the semen analysis results.[82,8] However grade of varicocele by itself should not be the sole indication for treatment.

Testicular volume

There is an abundance of literature confirming that varicocele is associate with testicular growth arrest in adolescents and varicocele repair results in testicular "catch-up" growth.[10,105,108,109] Testicular growth arrest with volume difference of more than 2 ml assessed by ultrasonography is the most common indication for treatment.[10] The development of secondary infertility is another strong argument for early varicocele repair as if left untreated the varicocele will not only effect testicular volume but also effects spermatogenesis.[6] A decrease in testicular volume is the best indicator for surgical correction of a varicocele. However, not every boy with a varicocele and testicular growth arrest will be infertile and there is still a need to search for a test which would better distinguish between adolescents with varicocele who will develop infertility and those who will remain fertile. In adult males the situation is a little simpler because the indications for surgery are usually established after 12 months of infertility confirmed by abnormal semen analysis and the presence of a varicocele. Obtaining a semen sample in adolescents is possible but difficult.

Gonadotropin releasing hormone (GnRH) stimulation test

Damage to germinal epithelium results in compensatory stimulation of the pituitary gland and subsequent increase in FSH and LH production by gonadotrophs. [111] Intravenous administration of GnRH stimulates the pituitary gland to release FSH and LH. FSH levels are elevated in any condition (like varicocele) effecting the integrity of germinal epithelium. [112] In theory GnRH stimulation test could distinguish between adolescents with a varicocele who have abnormal testicular functions and those who have normal spermatogenesis.[11] However clinical practice showed that the GnRH stimulating test is expensive, requires multiple serum samples and lacks the association between abnormal results, growth arrest and infertility.[113 , 114] An abnormal GnRH stimulating test was found in 30 % of adolescents with varicocele and was not correlated with atrophy or infertility. [115] Currently it seems that GnRH stimulation test has limited role in clinical evaluation of adolescent with a varicocele.

Pampiniform plexus veins diameter

Ultrasonographic measurement of pampiniform plexus veins diameter (PPVD) has been used to look for subclinical varicocele in adult infertile population when physical exam is inconclusive and to follow persistent varicocele but PPVD measurements are not useful in adolescents.[116,117]

Inhibin level

The serum inhibin levels reflect the integrity of the seminiferous tubule. However, there is not enough data to use serum inhibin levels in clinical decisions at this time.[68,74,118-120]

Currently, prophylactic surgery for every adolescent with varicocele is not advised since it would result in treatment of 15 % of adolescents.

In summary it seems that treatment should be offered to:

- adolescents with testicular growth arrest (2 SD from normal testicular growth curves, more than 2 cc of difference between left and right testicle)
- adolescents with abnormal semen analysis with high-grade varicocele
- adolescents with symptoms: pain, heaviness, swelling
- adolescents with bilateral varicoceles

TREATMENT OPTIONS

Treatment options in the management of adolescent with a varicocele originated from the practice of adult male infertility and subsequently were applied to the treatment of adolescents.

Varicocele repair can be done by surgical ligation and division of testicular veins or intravenous embolization of testicular veins.

Three open surgical approaches are currently used: subinguinal approach (Marmar), inguinal approach (Ivanissevich) and retroperitoneal approach (Palomo). Laparoscopic varicocele ligation has often been used in adults. Embolization techniques, regardless of embolizing material, can be divided into: antegrade (infusion through scrotal part of pampiniform plexus veins) and retrograde (catheter placed through femoral vein puncture) infusion.

The failure rate, frequency of complications, cost and outcome are important factors which need to be evaluated in choosing preferable treatment option in adolescent repair.(Table 2) It is important to remember that the majority of studies on varicocele repair relates to adult infertile males with varicocele and not adolescents.

TABLE 2
Surgical approach, complications and relative costs of varicocele repair.

Technique	Hydrocele rate	Failure rate	Cost	References
Retroperitoneal	7-10 %	9-11 % (artery sparing) <3 % (artery taking)	Low	121 10 12
Inguinal	3-7 %	9-12%	Low	123 129 147
Inguinal microscopic	< 1%	2.1 % 0.6%	Moderate	124 125
Laparoscopic	1.25 %	9 % 1.25 % in adolescents	High	148 149
Embolization		19 %	High	135

FAILURE RATE

Failure of treatment is defined here as a persistent or (rarely) recurrence of the varicocele after the repair and can occur in 9 % to 16 % of adolescents.[121] Persistence of the varicocele results in lack of "catch-up" testicular growth.[12] Most authors attribute the high recurrence rate to missed venous collaterals which run parallel to the main testicular vein. The collaterals can be quite difficult to identify and ligate separately from the testicular artery. Reported persistence rate using artery sparring retroperitoneal approach ranges from 3% to 11%.[12,121-123] Ligation of both testicular vein trunci and the artery has the advantage of a decreased persistence rate and does not result in testicular atrophy since the testis has collateral arterial blood supply from the cremasteric and deferential artery.[12,21] Atassi and colleagues achieved a persistence rate below 2 % in adolescents treated by high retroperitoneal ligation with testicular artery ligation.[12]

There is, however, some objection to simultaneous ligation of the testicular artery in men with previous inguinal surgery since there may be compromised blood supply from the cremasteric and deferential arteries. Interruption of the testicular artery in these patients has a high probability of

developing testicular atrophy. Also, subsequent vasectomy in patients with testicular artery division should be avoided since ligation of the vasal artery could result in testicular atrophy in the absence of the testicular artery.

The high persistence rate and postoperative hydrocele rate resulted in the development of microsurgical inguinal approaches for correction of the varicocele. Both subinguinal and inguinal microsurgical repair are used quite often in adults with varicocele and indeed offer lower persistence rates and a low incidence of postoperative hydrocele.[124-126] The low persistence rate using the microsurgical inguinal repair is attributed to ligation of all distended vein trunci and collaterals at the level of the deep inguinal ring.[124-126] Experience in microsurgical inguinal varicocele repair in adolescent is limited. Reports by Minevitch and Goldstein demonstrated a significantly lower rate of persistence and postoperative hydrocele.[127,128] The microscopic approach allows one to ligate only the veins leaving the lymphatic vessels intact what decreased the postoperative hydrocele rate to less than 1%.[125,126]

Laparoscopic varicocele repair with, and without artery sparing modifications seems to be suitable treatment technique especially since recent reports in adults showed a lower rate of persistence.[129-131] Laparoscopic surgery in the pediatric population is gaining acceptance but it bares the risk of significant complications like bowel perforation, major vascular injury, pneumothorax, and incisional hernia. There is not much experience with laparoscopic varicocele ligation in the adolescent population.[132-134]

Retrograde embolization, unfortunately is associated with an unacceptable high rate of persistence and is the most expensive of treatment techniques.[1] Possible explanation of such a high persistence rate of the varicocele after embolization can be attributed to the highly variable anatomy of the testicular venous drainage and technical difficulties.[135] Antegrade embolization is more often used to treat persistent varicoceles than as an initial treatment.[136-139]

Other options to decrease the rate of varicocele persistence are intraoperative venography and methylene blue injections.[27,123,140,141] Intraoperative venography in theory should facilitate ligation of all testicular vein trunci and decrease the rate of persistence. Hart recommends routine use of intraoperative venography because 16% of their 62 patients had missed venous vessels after initial venous ligation.[142] Similar conclusions, based on a decreased persistence rate, were also made by Levitt, Zaontz and Gill.[143-145] However Palmer and Kass reported no difference in their rate of varicocele persistence after repair with and without intraoperative venography.[121,123,141] Based on these studies intraoperative venography offer little benefit in the repair of adolescents with a varicocele.

Based on our review of the literature and the results of a survey of pediatric urologists in the United States high retroperitoneal ligation of the testicular artery and veins offers the best results in adolescents. Although currently the high retroperitoneal approach is a treatment of choice in adolescent varicocele, pediatric urologists should consider using the microscopic inguinal or subinguinal approach with arterial preservation.

CONCLUSIONS

The adolescent with a varicocele presents the clinician with an interesting and challenging problem. There is a great need for further basic research to help better select the patient who needs surgical correction of his varicocele.

We have outlined recommendations which can be used in everyday practice. Each clinician needs to make his/her own decisions regarding who, when and how to treat the adolescent with varicocele.

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This article should be referenced as follows:

Paduch, D.A. and Skoog, S.J. (2004) Diagnosis, evaluation and treatment of adolescent varicocele. *TheScientificWorldJOURNAL* **4 (S1)**, 263–278.

Handling Editor:

Anthony Atala, Principle Editor for *Urology* — a domain of *TheScientificWorldJOURNAL*.

Heat shock factor Y chromosome (HSFY) mRNA level predicts the presence of retrievable testicular sperm in men with nonobstructive azoospermia

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Objective: To evaluate heat shock factor Y chromosome (HSFY) mRNA as a biomarker for the presence of retrievable testicular sperm.
Design: Case-control study.
Setting: Academic medical center.
Patient(s): Men with nonobstructive azoospermia (NOA).
Intervention(s): Testicular tissue from men with successful or failed testicular sperm extraction was evaluated with qunatitative real-time polymerase chain reaction (qRT-PCR) for expression of HSFY mRNA.
Main Outcome Measure(s): Area under the receiver operating characteristic curve (AUC), sensitivity, specificity, and probability of sperm retrieval based on HSFY testing.
Result(s): We found higher HSFY mRNA expression in testicular tissue from NOA patients in whom sperm were successfully retrieved compared with those in whom sperm were not found, with good discrimination between the groups in all histologic variants of NOA (AUC 0.89 overall, 0.98 for patients with Sertoli cell only [SCO] histology, 0.90 for patients with maturation arrest [MA] histology). Sensitivity and specificity were, respectively, 67% and 93% overall, 92% and 100% for SCO patients, and 67% and 92% for MA patients. The probabilities of sperm retrieval for HSFY-positive and -negative patients were, respectively, 93% and 31% overall, 100% and 7% for SCO patients, and 91% and 32% for MA patients.
Conclusion(s): Detection of HSFY mRNA expression by qRT-PCR has promising application in the evaluation and counseling of men with NOA before attempted sperm retrieval surgery. (Fertil Steril® 2011;96:303 8. ©2011 by American Society for Reproductive Medicine.)

Key Words: Nonobstructive azoospermia, sperm retrieval, heat shock factor Y chromosome (HSFY), testicular biopsy, male infertility

The optimal management of nonobstructive azoospermia (NOA) is testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI). However, sperm are absent in the testes of many men with NOA, and preoperative clinical parameters cannot reliably predict the presence of retrievable testicular sperm. Microdissection TESE appears to provide sperm retrieval rates (SRRs) as high or higher than with conventional TESE or percutaneous sperm retrieval procedures (1 4), but it nonetheless fails in 37% 65% of patients with NOA (5). Men in whom sperm are not retrieved needlessly incur some risk of morbidity, psychologic stress, and financial expense. Furthermore, at centers such as ours that perform ICSI with freshly retrieved sperm, female partners of men with NOA who fail sperm retrieval may undergo unnecessary ovarian stimulation in cases when the couple is unwilling to use donor sperm. Such cases are relatively frequent. According to one study, only 43% of American subfertile couples consider donor insemination a viable option (6).

Received February 10, 2011; revised May 10, 2011; accepted May 18, 2011; published online June 15, 2011.
P.J.S. has nothing to disclose. A.M. has nothing to disclose. P.N.S. has nothing to disclose. D.A.P. has nothing to disclose.
Supported by grants from the Research Scholar program of the American Urological Association Foundation and the Frederick J. and Theresa Dow Wallace Fund of the New York Community Trust (P.J.S.).
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In our laboratory, we previously observed low testicular mRNA expression of the AZFb gene HSFY in patients with NOA who fail microdissection TESE, including patients with both early and late maturation arrest (MA), suggesting the potential role of HSFY as a marker of complete spermatogenesis (7). Our objective was to evaluate detection of HSFY mRNA expression by quantitative real-time polymerase chain reaction (qRT-PCR) on a random testicular tissue sample as a diagnostic test to predict the presence of retrievable testicular sperm in men with NOA.

MATERIALS AND METHODS

Patients

The Institutional Review Board approved this study. The study population included 54 men with NOA who underwent microdissection TESE. Nine men with obstructive azoospermia (OA) who underwent testicular biopsy during sperm retrieval surgery served as control subjects. Patients were selected based on availability of well preserved testicular biopsies for pathologic analysis and availability of testicular tissue for research. Preoperative evaluation included history, measurement of testis size with an orchidometer, semen analysis, serum FSH level, karyotyping, and Y chromosome microdeletion testing.

Sperm Retrieval

Azoospermia was confirmed on the day of sperm retrieval by microscopic analysis of ejaculated semen after centrifugation. Microdissection TESE was performed with the use of an operating microscope and a transverse incision in the tunica albuginea until sperm were found or the entire volume of

testicular tissue was dissected (1). Extracted testicular tissue from each dissected region of the testis was immediately placed into a small volume of fluid and mechanically disrupted with sharp scissors and sequential passes through a 24-gauge angiocatheter. A small aliquot (10 microliters) of the testis tissue suspension was then placed on a slide and cytologically examined in the operating room for the presence of sperm by an experienced andrologist to direct the extent and duration of surgery. This slide was discarded after analysis. The remainder of each testis tissue suspension was subsequently analyzed in the andrology laboratory for identification of sperm. Microdissection TESE was considered to be successful if one or more sperm were found in the laboratory that were morphologically acceptable for ICSI.

Tissue Acquisition for Histopathology and qRT-PCR

Diagnostic testicular biopsies and seminiferous tubular tissue for research were taken during microdissection TESE after the tunica albuginea was widely opened. Randomly selected pieces of undisturbed seminiferous tubular tissue measuring 5–10 mm in greatest dimension were sharply excised. One piece of tissue was placed gently into Bouin's solution for pathological analysis. Tissue for research was placed without media into a cryovial, immediately snap frozen in liquid nitrogen, and stored at 80°C.

Pathologic Analysis

Histopathologic analysis was performed as previously described (8). Sections were stained with hematoxylin and eosin and examined with a light microscope under ×100–400 magnification. Biopsies were classified according to the most advanced pattern of spermatogenesis observed anywhere within the tissue biopsied. We classified biopsies as Sertoli cell only (SCO) when germ cells were completely absent ("pure" SCO), as MA when germ cells were identified anywhere in the biopsy specimen but elongated spermatids were completely absent, and as hypospermatogenesis (HS) when any condensed oval sperm heads were identified.

Quantitative Analysis of HSFY mRNA Expression

Frozen seminiferous tubular tissue for RNA extraction was thawed, weighed, and homogenized. We extracted RNA with Trizol LS Reagent (Invitrogen). To remove contamination with genomic DNA, extracted RNA was incubated with RNase-free DNase for 30 minutes (Qiagen) and purified with an RNA-binding spin column (RNeasy Mini Kit; Qiagen). We measured RNA concentration spectrophotometrically at 260 nm, and purity was confirmed by measurement of the A260/A280 ratio. We synthesized cDNA from 1 μg purified total RNA with random hexamer primers with the use of the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). cDNA was stored at 20°C until use.

HSFY transcript variant 1 mRNA level was measured with the use of dual-color multiplex qRT-PCR with the Universal Probe Library (UPL) hydrolysis probe set on a Light Cycler 480 instrument (Roche Diagnostics). Porphobilinogen deaminase (PBGD) was used as the housekeeping gene for relative quantification based on observations in our laboratory of consistent PBGD expression in human testis regardless of histology (data not shown). The multiplex assay was designed with the use of the UPL Assay Design Center (http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp). HSFY transcript variant 1 mRNA was detected with the forward and reverse primers 5′-GTCAATGAGGCTCCATATCGT-3′ and 5′-GATCGTAGGCATTTGCA ACC-3′, respectively, and UPL Probe #40. PBGD mRNA was detected with a proprietary Human PBGD Gene Assay (Roche Diagnostics).

All qRT-PCR reactions were run in duplicate on 96-well plates. The 20-μL reaction mixture contained 5 μL 1:5 diluted cDNA and 200 nmol/L UPL probe, 200 nmol/L PBGD probe, 200 nmol/L forward and reverse primers for HSFY, 500 nmol/L forward and reverse primers for PBGD, and 1× Lightcycler 480 Probes Master Mix. The cycle protocol was as follows: denaturation at 95°C for 10 minutes, 45 cycles of 95°C for 10 seconds and 60°C for 30 seconds, and a cooling cycle to 55°C. HSFY/PBGD expression ratio was determined with Lightcycler 480 Relative Quantification software (Roche Diagnostics). Standard curves were generated during each PCR run for both HSFY and PBGD by running the multiplex reaction in triplicate with serially diluted cDNA from a patient with OA. Crossing points were determined by the second derivative maximum method. PCR efficiency corrections were applied by the software based on the standard curves and the calculated efficiencies for the HSFY (1.7) and PBGD (1.6) reactions. We confirmed assay validity by analysis of the HSFY/PBGD expression ratio in testicular tissue from a patient with an AZFb deletion that included loss of both copies of HSFY. HSFY/PBGD expression ratio in this patient was negligible.

Statistical Analysis

Statistical analysis was performed with Graphpad Prism Version 5.0c. Serum FSH, average testicular volume, age, and mean HSFY/PBGD ratio were analyzed in relation to microdissection TESE outcome with the use of the Mann-Whitney test. The performance characteristics of HSFY/PBGD expression ratio to predict the presence of retrievable sperm were determined by receiver operating characteristic (ROC) curve analysis. Positive and negative likelihood ratios were calculated as sensitivity divided by (1 specificity) and (1 sensitivity) divided by specificity, respectively (9). The probability of sperm retrieval based on HSFY/PBGD testing was determined with Fagan's nomogram for Bayes theorem (10) using the calculated likelihood ratios and the overall and histology-specific sperm retrieval rates at our institution from 1999 to 2010.

RESULTS

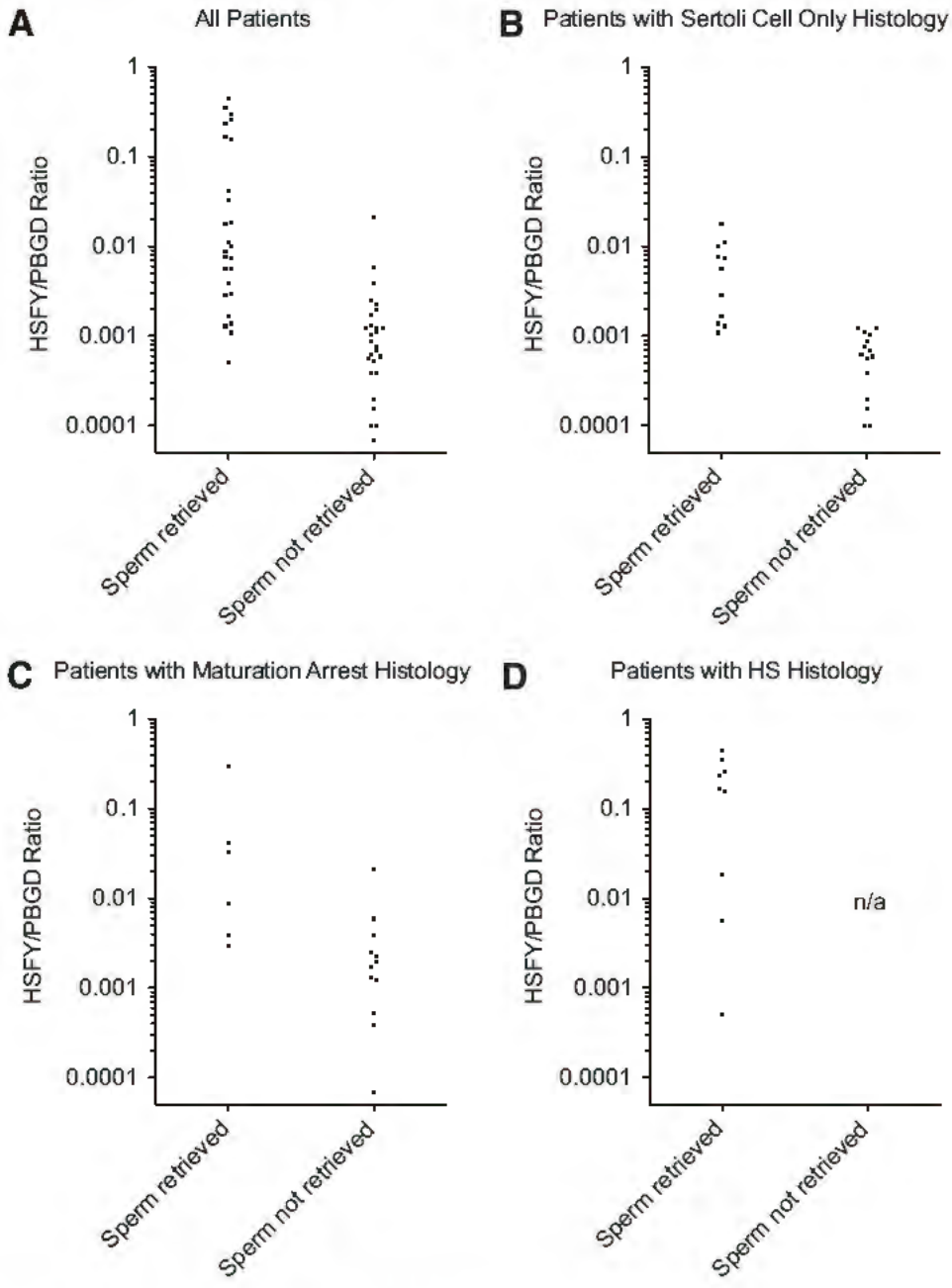
The 54 patients in the study population included 44 men with unexplained NOA, five with a history of cryptorchidism, three with prior chemotherapy exposure, one with Klinefelter syndrome, and one with an AZFc deletion. Sperm were retrieved in 27/54 cases (50%). Histopathology in the successful retrieval group was SCO, MA, and HS in 12, 6, and 9 patients, respectively. The failed-retrieval group included 15 patients with SCO histology and 12 with MA. There was no difference in mean age between the two groups [34.6 (95% confidence interval [CI] 31.4 37.8) years vs. 33.4 (95% CI 31.6 35.2) years; P=.910]. Patients in whom sperm retrieval was successful had higher mean FSH values [23.9 (95% CI 18.9 28.8) IU/L vs. 16.7 (95% CI 1.9 21.5) IU/L; P=.016] and lower mean testicular volumes [9.0 (95% CI 7.0 11.0) mL vs. 11.8 (95% CI 10.1 13.5) mL; P=.037] compared with patients in whom retrieval failed.

The mean weight of testis tissue used for RNA extraction was 78.1 (range 10 240) mg. Sufficient RNA for analysis was extracted in all cases. The mean HSFY/PBGD expression ratio in the control patients with OA was 0.673 (standard error 2.56×10^{2}, range 0.145 0.946). The HSFY/PBGD expression ratios in NOA patients in relation to microdissection TESE outcome are presented in Figure 1 and Table 1. We observed higher HSFY/PBGD expression ratios in patients with successful microdissection TESE compared with those with failed microdissection TESE within the overall study population, as well as within the SCO and MA subgroups. Comparison was not possible for the HS subgroup, because sperm were retrieved in all cases. However, HSFY/PGBD expression ratios were high or very high in 8/9 patients with HS histology (Fig. 1). The one patient with HS who had a low expression ratio was a patient with an AZFc deletion, in whom diagnostic biopsy revealed that 98% of tubules were SCO pattern and 2% of tubules contained very rare mature sperm.

The areas under the ROC curves (AUCs) for the entire cohort, the SCO subgroup, and the MA subgroup were 0.89, 0.98, and 0.90, respectively (Fig. 2). The optimal cutoff values for a positive HSFY/PBGD test were $>4.48 \times 10^{3}$ for the entire cohort, $>1.20 \times 10^{3}$ for the SCO subgroup, and $>7.40 \times 10^{3}$ for the MA subgroup. Performance characteristics of HSFY/PBGD expression ratio to predict the presence of retrievable testicular sperm are presented in Table 2.

FIGURE 1

Scatter plots showing HSFY/PBGD expression ratios (**A**) for every nonobstructive azoospermia patient in the study and (**B–D**) for every patient within each histologic subgroup (HS = hypospermatogenesis). Each dot represents the HSFY/PBGD expression ratio measured in one patient.



Stahl. HSFY predicts sperm retrieval in nonobstructive azoospermia. Fertil Steril 2011.

DISCUSSION

Patients and physicians accept the high failure rates of testicular sperm retrieval in NOA for two reasons. First, genetic parenthood is such a critical quality of life issue that affected couples are often willing to assume the risks and costs of TESE despite the possibility of failure of sperm retrieval. Second, the performance characteristics of available clinical tests to predict TESE outcome are insufficient in almost all cases to preclude an attempt at testicular sperm retrieval. Neither serum hormone assays, such as FSH and inhibin B, nor noninvasive assessments, such as testicular volume, alter the probability of sperm retrieval sufficiently to direct clinical management (5).

The only noninvasive method that is helpful in selecting patients for microdissection TESE is Y microdeletion testing. Y microdeletions that involve loss of the complete AZFa or AZFb regions are

TABLE 1

HSFY/PBGD expression ratios in NOA patients with respect to mTESE outcome.

	NOA, sperm retrieved (n = 27)	NOA, sperm not retrieved (n = 27)	*P* value[a]
All patients	$7.76 \times 10^{-2} \pm 2.47 \times 10^{-2}$	$1.90 \times 10^{-3} \pm 8.00 \times 10^{-4}$	<.0001
SCO	$5.70 \times 10^{-2} \pm 1.5 \times 10^{-3}$	$6.00 \times 10^{-4} \pm 1.00 \times 10^{-4}$	<.0001
MA	$6.52 \times 10^{-2} \pm 4.76 \times 10^{-2}$	$3.60 \times 10^{-3} \pm 1.60 \times 10^{-3}$	.0076
HS	$1.82 \times 10^{-1} \pm 5.26 \times 10^{-2}$	n/a	n/a

Note: Values are given as mean ± SEM. HS hypospermatogenesis; MA maturation arrest; NOA nonobstructive azoospermia; SCO Sertoli cells only.
[a] Mann-Whitney test.

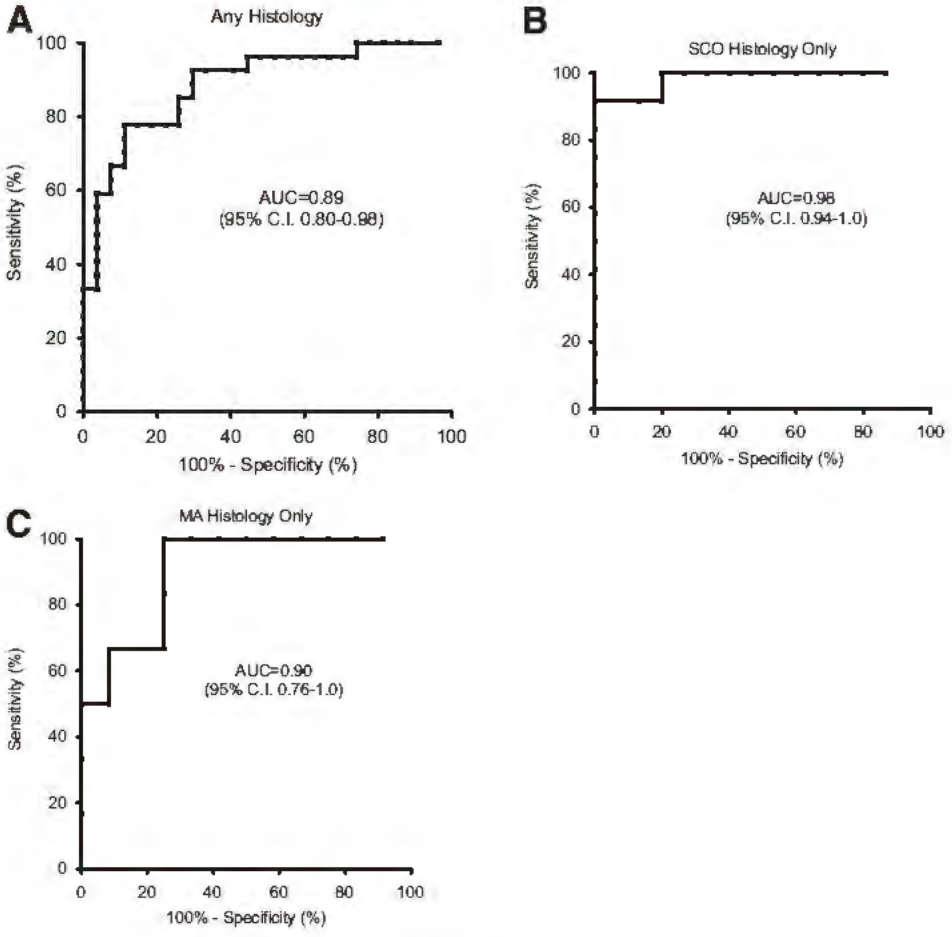
Stahl. HSFY predicts sperm retrieval in nonobstructive azoospermia. Fertil Steril 2011.

incompatible with sperm production and are found in up to 6% of American men with NOA (11). We do not recommend microdissection TESE to these patients.

Open or percutaneous testicular biopsy for histologic assessment is more informative than noninvasive testing and allows for therapeutic sperm retrieval in some cases. Despite having a relationship with microdissection TESE outcome (8, 12), testicular histology does not change the probability of sperm retrieval enough to affect clinical management for men with NOA. Reported SRRs in men with SCO histology, the least favorable histologic diagnosis, are 24% 48% (4). In our experience, nearly all of these patients elect to proceed with sperm retrieval, given the possibility of successful

FIGURE 2

Receiver operating characteristic curves for HSFY mRNA detection by quantitative real-time polymerase chain reaction to predict the presence of retrievable testicular sperm among the (A) overall study population and the (B) Sertoli cell–only (SCO) and (C) meturation arrest (MA) subgroups.



Stahl. HSFY predicts sperm retrieval in nonobstructive azoospermia. Fertil Steril 2011.

TABLE 2

Test characteristics of HSFY/PBGD ratio to predict sperm retrieval based on the optimal cutoff values selected for the overall study population, and the SCO and MA subgroups.

	Sensitivity	Specificity	Positive likelihood ratio	Negative likelihood ratio	Institutional sperm retrieval rate per microdissection TESE (1999–2010)	Probability of sperm retrieval for an individual patient[a]
All patients	66.7%	92.6%	9.0	0.36	56.6% (606/1070)	HSFY+: 93% HSFY−: 31%
SCO	91.7%	100%	∞	0.08	48.3% (253/524)	HSFY+: 100% HSFY−: 7%
MA	66.7%	91.7%	3.3	0.36	56.1% (92/164)	HSFY+: 91% HSFY−: 32%
HS	n/a	n/a	n/a	n/a	94.3% (149/158)	n/a

Note: TESE = testicular sperm extraction; other abbreviations as in Table 1.

[a] Derived using Fagan's nomogram for Bayes theorem (9).

Stahl. HSFY predicts sperm retrieval in nonobstructive azoospermia. Fertil Steril 2011.

treatment. In recognition of the minimal clinical impact of testicular histology among men with NOA, our and other centers have abandoned the routine use of preoperative testicular biopsies in these men. However, routine use of preoperative testicular biopsy may be worth reconsidering in the context of new molecular diagnostic tests, such as HSFY mRNA detection with qRT-PCR, that significantly improve the prognostic utility of testicular biopsy.

Detection of candidate spermatogenesis-specific mRNA in testis tissue has been used previously with some success to detect occult foci of spermatogenesis in men with NOA (13 15). These studies evaluated detection of CDY1 (13), protamine, and cyclin (14, 15) mRNA transcripts to predict TESE outcome, bnt they were all limited by the inability to discriminate between patients with SCO histology who did and did not harbor retrievable testicular sperm. In contrast, in the present pilot study we demonstrated that the level of HSFY transcript variant 1 mRNA is highly predictive of microdissection TESE outcome in men with all histologic variants of NOA, particularly in men with SCO histology.

Two copies of the HSFY gene are present within palindrome P4 of the AZFb region of the Y chromosome (16). These genes encode three different mRNA transcripts that are expressed in human testis. Only the protein translated from transcript variant 1 contains a heat shock factor like DNA-binding domain (17), suggesting that this mRNA is the critical HSFY transcript. Although the function of HSFY is not presently understood, it is expressed in human germ cells and Sertoli cells (18) and likely acts by moderating expression of heat shock proteins, which serve as important transcription factors.

It is interesting that HSFY mRNA expression seems to specifically reflect the presence of mature germ cells in the testis, despite the fact that expression is present within Sertoli cells and is not germ cell specific. Several explanations for this observation are feasible. Sertoli cell expression of HSFY mRNA may depend on cellular crosstalk with mature germ cells. Alternatively, expression in Sertoli cells may be a driver of meiosis or spermiogenesis. The latter hypothesis is supported by the observation that men with AZFb deletions, in which both copies of HSFY are deleted, invariably present with nonobstructive azoospermia and diffuse maturation arrest.

The HSFY/PBGD expression ratio measured by qRT-PCR may be used in combination with testicular histopathology and historical SRRs from the treating center to counsel individual patients about their chances of sperm retrieval. The benefit of HSFY testing is illustrated by considering the case of a patient with idiopathic NOA whose diagnostic biopsy shows SCO pattern. In the absence of HSFY testing, we would counsel this patient that his chance of successful sperm retrieval is 35% 40% (19). Nearly all such men elect to proceed with microdissection TESE, given the reasonable chance of success. However, if such a patient tested positive for HSFY expression, he could be counseled that the chance of sperm retrieval is close to 100%. Conversely, if he were to test negative the estimated chance of sperm retrieval would be 7%. Considering the 29% 40% ongoing pregnancy rates reported in IVF-ICSI cycles using testicular sperm from men with NOA (20), the chance of achieving an ongoing pregnancy in such a patient would be 2% 3%. Though some men might still elect to proceed in this scenario, the medical risks and financial expenses may not be justified.

Despite its promising performance in this study, the clinical applicability of HSFY testing remains to be determined. First, it is important to recognize that the calculated posttest probabilities of sperm retrieval based on HSFY testing were derived from our institutional SRRs and may differ considerably at institutions with different

SRRs. The efficacy of microdissection TESE depends on many factors, including degree of microdissection, experience of the operating surgeon, and experience of the andrology laboratory. Therefore, the clinical utility of HSFY testing needs to be evaluated at other centers. Furthermore, we obtained testicular tissue for RNA extraction by microsurgical open testicular biopsy under general anesthesia. This approach may be overly invasive and expensive to be a practical cost-effective method for assessment of the prognosis for sperm retrieval. Because the tissue obtained in this study was obtained from random sites with limited tissue, it is likely that samples could be obtained percutaneously in the outpatient setting under local anesthesia. Patients could undergo one simple office-based procedure during which limited testicular tissue could be procured for simultaneous histologic assessment and HSFY testing, and during which a limited attempt could be made at therapeutic sperm retrieval.

Large-needle percutaneous aspiration biopsy is simple and on average yields 385 mg of testicular tissue (21) (far more tissue than was required for the present study). Fine-needle aspiration (FNA) is performed with a smaller-bore needle but is a more complex procedure which typically requires multiple passes with the needle in multiple directions. These procedures are best performed by an experienced urologist, owing to the risk of disrupting centripetal arterioles within the testicular parenchyma, which may lead to clinically significant bleeding and subsequent development of intratesticular fibrosis. Further studies are needed to evaluate the adequacy of large-needle aspiration biopsy and FNA for detection of testis-specific RNA transcripts.

Despite the potential limitations of the present analysis, the results suggest that measurement of HSFY mRNA in testicular tissue may significantly improve our ability to counsel patients with NOA and to select patients most likely to have sperm found with microdissection TESE. We hope that this research will eventually help to minimize the number of futile attempts at testicular sperm retrieval and to increase the SRR in NOA.

Acknowledgments: Dr. Marc Goldstein served as a scientific advisor.

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PLOS | GENETICS

Human Spermatogenic Failure Purges Deleterious Mutation Load from the Autosomes and Both Sex Chromosomes, including the Gene *DMRT1*

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Abstract

Gonadal failure, along with early pregnancy loss and perinatal death, may be an important filter that limits the propagation of harmful mutations in the human population. We hypothesized that men with spermatogenic impairment, a disease with unknown genetic architecture and a common cause of male infertility, are enriched for rare deleterious mutations compared to men with normal spermatogenesis. After assaying genomewide SNPs and CNVs in 323 Caucasian men with idiopathic spermatogenic impairment and more than 1,100 controls, we estimate that each rare autosomal deletion detected in our study multiplicatively changes a man's risk of disease by 10% (OR 1.10 [1.04–1.16], $p<2\times10^{-3}$), rare X-linked CNVs by 29%, (OR 1.29 [1.11–1.50], $p<1\times10^{-3}$), and rare Y-linked duplications by 88% (OR 1.88 [1.13–3.13], $p<0.03$). By contrasting the properties of our case-specific CNVs with those of CNV callsets from cases of autism, schizophrenia, bipolar disorder, and intellectual disability, we propose that the CNV burden in spermatogenic impairment is distinct from the burden of large, dominant mutations described for neurodevelopmental disorders. We identified two patients with deletions of *DMRT1*, a gene on chromosome 9p24.3 orthologous to the putative sex determination locus of the avian ZW chromosome system. In an independent sample of Han Chinese men, we identified 3 more *DMRT1* deletions in 979 cases of idiopathic azoospermia and none in 1,734 controls, and found none in an additional 4,519 controls from public databases. The combined results indicate that *DMRT1* loss-of-function mutations are a risk factor and potential genetic cause of human spermatogenic failure (frequency of 0.38% in 1306 cases and 0% in 7,754 controls, $p=6.2\times10^{-5}$). Our study identifies other recurrent CNVs as potential causes of idiopathic azoospermia and generates hypotheses for directing future studies on the genetic basis of male infertility and IVF outcomes.

Citation: Lopes AM, Aston KI, Thompson E, Carvalho F, Gonçalves J, et al. (2013) Human Spermatogenic Failure Purges Deleterious Mutation Load from the Autosomes and Both Sex Chromosomes, including the Gene *DMRT1*. PLoS Genet 9(3): e1003349. doi:10.1371/journal.pgen.1003349

Editor: Edward Hollox, University of Leicester, United Kingdom

Received June 14, 2012; **Accepted** January 17, 2013; **Published** March 21, 2013

Funding: This work was partially funded by the Portuguese Foundation for Science and Technology FCT/MCTES (PIDDAC) and co-financed by European funds (FEDER) through the COMPETE program, research grant PTDC/SAU-GMG/101229/2008. IPATIMUP is an Associate Laboratory of the Portuguese Ministry of Science,

Technology, and Higher Education and is partially supported by FCT. AML is the recipient of a postdoctoral fellowship from FCT (SFRH/BPD/73366/2010). CO is supported by a grant from the United States National Institutes of Health (R01 HD21244), JDS is supported by Damon Runyon Clinical Investigator Award, Alex's Lemonade Stand Foundation Epidemiology Award, and the Eunice Kennedy Shriver Children's Health Research Career Development Award NICHD 5K12HD001410. Support for humans studies and specimens were provided by the NIH/NIDDK George M. O'Brien Center for Kidney Disease Kidney Translational Research Core (P30DK079333) grant to Washington University. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Male infertility is a multifaceted disorder affecting nearly 5% of men of reproductive age. In spite of its prevalence and a considerable research effort over the past several decades, the underlying cause of male infertility is uncharacterized in up to half of all cases [1]. Some degree of spermatogenic impairment is present for most male infertility patients, and, in its most severe form, manifests as azoospermia, the lack of detectable spermatozoa in semen, or oligozoospermia, defined by the World Health Organization as less than 15 million sperm/mL of semen. Spermatogenesis is a complex multistep process that requires germ cells to (a) maintain a stable progenitor population through frequent mitotic divisions, (b) reduce ploidy of the spermatogonial progenitors from diploid to haploid through meiotic divisions, and (c) assume highly specialized sperm morphology and function through spermiogenesis. These steps involve the expression of thousands of genes and carefully orchestrated interactions between germ cells and somatic cells within the seminiferous tubules [2]. It is likely that a large proportion of idiopathic cases of spermatogenic failure are of uncharacterized genetic origin, but measuring the heritability of infertility phenotypes has been challenging.

Known genetic causes of non-obstructive azoospermia (NOA) include deletions in the azoospermia factor (AZF) regions of the Y chromosome [3], Klinefelter's syndrome [4], and other cytogenetically visible chromosome aneuploidies and translocations [5]. Beyond these well-established causes, which are observed in 25–30% of cases, the genetic architecture of spermatogenic impairment is currently unknown. One might expect *a priori* that rare or *de novo*, large effect mutations will be the central players in genetic infertility, and indeed other primary infertility phenotypes like disorders of gonadal development, isolated gonadotropin-releasing hormone deficiency, and globozoospermia, a disorder of sperm morphology and function, appear to be caused by essentially Mendelian mutations operating in a monogenic or oligogenic fashion [6,7,8]. Similarly, recurrent mutations of the AZF region on the Y chromosome are either completely penetrant (AZFa, AZFb/c) or highly penetrant (AZFc) risk factors for azoospermia. Our working model at the start of this study was that additional "AZF-like" loci existed in the genome, either on the Y chromosome or elsewhere, and that, much like recent progress in the analysis of developmental disorders of childhood, a large number of causal point mutations and submicroscopic deletions could be revealed in idiopathic cases by the appropriate use of genomic technology.

In this paper, we employ oligonucleotide SNP arrays as discovery technology to conduct a whole-genome screen for two rare genetic features in men with spermatogenic failure. First, we extract and analyze the probe intensity data to find rare copy number variants (CNVs). A growing number of CNVs have been associated with a host of complex disease states [9] including neurological disorders [10,11,12,13], several autoimmune diseases [14,15], type 2 diabetes [16], cardiovascular disease [17], and cancer [18,19,20,21]. Now, a role for CNVs in male infertility is beginning to emerge [22,23,24,25].

As a second approach to identify rare genetic variants, we use a population genetics modeling framework to identify large homozygous-by-descent (HBD) chromosome segments that may harbor recessive disease alleles. When applied to consanguineous families, so-called "HBD-mapping" has been an unequivocal success in identifying the location of causal variants for simple recessive monogenic diseases [26]. HBD analysis can also be used to screen for the location of rare variants in common disease case-control studies of unrelated individuals, using either a single-locus association testing framework or by testing for an autozygosity burden, frequently referred to as "inbreeding depression": an enrichment of size or predicted functional impact of HBD regions aggregated across the genome. This approach has produced results for a growing list of common diseases, including schizophrenia [27], Alzheimer's disease [28], breast and prostate cancer [29].

In this study, we screened three cohorts of men with idiopathic spermatogenic failure in an attempt to identify rare, potentially causal mutations, and to better understand the genetic architecture of the disease (Table 1). We found a genomewide enrichment of large, rare CNVs in men with spermatogenic failure compared to normozoospermic or unphenotyped men (controls). We also identify a number of cases with unusual patterns of homozygosity, possibly the result of recent consangoineous matings. Our results show that spermatogenic output is a phenotype of the entire genome, not just the Y chromosome, place spermatogenic failure firmly among the list of diseases that feature a genomewide burden of rare deleterious mutations and provide a powerful organizing principle for understanding male infertility.

Results

First, we attempted to find evidence for undiscovered dominant causes of spermatogenic failure by studying the genomewide distribution of CNVs in our primary cohort from Utah: 35 men with idiopathic non-obstructive azoospermia, 48 men with severe oligozoospermia, and 62 controls with normal semen analysis. All cases had previously tested negative when screened for canonical Y chromosome deletions. Samples were assayed with an Illumina 370K oligonucleotide array that provides both SNP and CNV content. There was no detectable difference in the average number of CNVs called per sample among the three groups (mean = 20, azoospermic; 19.5, oligozospermic; 20, normozoospermic), however, the majority of variants (61% on average) in any one sample were common polymorphisms.

Rare CNV burden is a feature of spermatogenic failure

When restricting our analysis to CNVs with a call frequency of less than 5%, a subset likely to be enriched for pathogenic events,

Author Summary

Infertility is a disease that prevents the transmission of DNA from one generation to the next, and consequently it has been difficult to study the genetics of infertility using classical human genetics methods. Now, new technologies for screening entire genomes for rare and patient-specific mutations are revolutionizing our understanding of reproductively lethal diseases. Here, we apply techniques for variation discovery to study a condition called azoospermia, the failure to produce sperm. Large deletions of the Y chromosome are the primary known genetic risk factor for azoospermia, and genetic testing for these deletions is part of the standard treatment for this condition. We have screened over 300 men with azoospermia for rare deletions and duplications, and find an enrichment of these mutations throughout the genome compared to unaffected men. Our results indicate that sperm production is affected by mutations beyond the Y chromosome and will motivate whole-genome analyses of larger numbers of men with impaired spermatogenesis. Our finding of an enrichment of rare deleterious mutations in men with poor sperm production also raises the possibility that the slightly increased rate of birth defects reported in children conceived by *in vitro* fertilization may have a genetic basis.

we observed pronounced differences among groups (Table S1). Azoospermic and oligozoospermic men have nearly twice the amount of deleted sequence genomewide when compared to controls ($p = 1.7\times10^{-4}$, Wilcoxon rank sum test), and a nonsignificant 12% increase in the number of deletions per genome. When examining the even more restricted set of rare CNVs larger than 100 kb (Dataset S1), these associations are more pronounced: the rate of deletions in cases was twice that of controls (1.12 vs. 0.55, $p = 9.7\times10^{-4}$) and the amount of deleted sequence 2.6 times greater in cases ($p = 8.8\times10^{-4}$).

In order to replicate these initial findings, we assayed two additional cohorts – one group of 61 Caucasian men with severe spermatogenic impairment and 100 ethnicity-matched, unphenotyped controls, both collected at Washington University in St. Louis (WUSTL), and a larger case cohort of 179 Caucasian men with idiopathic azoospermia, primarily from medical practices in Porto, Portugal, matched to an unphenotyped control set of 974 Caucasian men collected by the UK National Blood Service (NBS, [30]). Although using different array platforms (Text S1), we observed replication of our initial association (Table S2 and Table S3); in the WUSTL cohort a 20% increase in the rate ($p<0.05$) and in the Porto cohort a 31% increase in rate ($p<5\times10^{-3}$). We excluded several artifactual explanations for this burden effect, including specific batch phenomena or population structure (Text S1, Figures S1, S2, S3, S4, S5). To better characterize these genomewide signals, we set out to search for clustering of pathogenic mutations on specific chromosomes.

We focused first on the Y chromosome as it is the location of most known mutations modulating human spermatogenesis (Figure 1, Figure S6). Y-linked microdeletions of the AZFa, AZFb, and AZFc regions are well-established causes of spermatogenic impairment, and thus we excluded from this study cases with AZF microdeletions visible by STS PCR. In the array data, we found no significant difference in the frequency of rare Y deletions between case and controls groups; however rare duplications were more abundant in Porto cases compared to the NBS controls (a 3-fold enrichment in Porto cohort, $p = 1.9\times10^{-3}$). We could classify the majority (>90%) of our samples to major Y haplogroups using SNP genotypes (Text S1), and, as expected, most of these samples fall into the two most common European haplogroups: I (22%) and R (70%). The observed duplication burden was not an artifact of differences in major Y haplogroup frequency between cases and controls, as association was essentially unchanged when only considering samples with haplogroup R1 ($p = 3.3\times10^{-3}$). Due to low probe coverage, only one Y-linked duplication was called in the Utah cohorts (in a control individual) and two in the WUSTL cohort (both in cases), so this burden of Y duplications was not replicated.

Next we turned to the X chromosome, which is highly enriched for genes transcribed in spermatogonia [31]. In the Utah cohorts there were 71 gains and losses with a frequency of less than 5% on the X chromosome, cumulatively producing three times as much aneuploid sequence in azoospermic and oligozoospermic men compared with normozoospermic men (89 kb/person azoo, 45 kb/person oligo, 27 kb/person normozoospermic men, all cases versus controls $p<0.03$). This burden was strongly replicated in the Porto samples, which displayed a 1.6 fold enrichment of rare CNV on the X ($p = 5\times10^{-4}$) and the WUSTL samples (31% of cases with a rare X-linked CNV versus 16% of controls, $p = 0.02$ by permutation).

The genome-wide signal of CNV burden was not driven solely by sex chromosome events: considering only autosomal mutations in Utah samples there was an enrichment of aneuploid sequence in large deletions in azoospermic men (268 kb/person) and oligozoospermic men (308 kb/person) compared to control men (189 kb/person, $p = 9.8\times10^{-3}$), and an enrichment in the rate of deletions in all cases when considering just events >100 kb (1.9 fold enrichment, $p = 6\times10^{-3}$). In the Porto cohort, there was modest evidence for a higher rate of rare deletions of all sizes in azoospermic men (1.27 fold enrichment, not significant) as well as an increase in total amount of deleted sequence (345 kb/case vs. 258 kb/control, $p<0.003$).

In order to cleanly summarize our findings across all cohorts, we fit logistic regression models for each cohort, regressing case status onto CNV count for different classes of CNV. We also fit a linear mixed-effects logistic regression model to the total dataset for each CNV class, treating cohort as a random factor (Figure 1). In each regression model we controlled for population structure by including eigenvectors from a genomewide principal components analysis (Methods). On the basis of the combined analysis, we estimate that each rare autosomal deletion multiplicatively changes the odds of spermatogenic impairment by 10% (OR 1.10 [1.04–1.16], $p<2\times10^{-3}$), each rare X-linked CNV (gain or loss) by 29%, (OR 1.29 [1.11–1.50], $p<1\times10^{-3}$) and each rare Y-linked duplication by 88% (OR 1.88 [1.13–3.13], $p<0.03$).

Locus-specific analyses

Deletions of the AZF regions of the Y chromosome are often mediated by non-allelic homologous recombination (NAHR) between segmental duplications and are the most common known cause of spermatogenic failure. Because of their prognostic power and high rate of recurrence in the population, screening for AZF deletions is a standard part of the clinical workup for azoospermia. It would be of high clinical value if additional azoospermia susceptibility loci with significant recurrence rates could be identified.

We screened all cohorts for large (>100 kb) rearrangements flanked by homologous segmental duplications capable of generating recurrent events by NAHR [32]. There was no significant enrichment of gains or losses in cases across these hotspot regions when considered as an aggregate. Due to small sample sizes we

Table 1. Case and control cohorts used in the study.

Cases					Controls			
Center	Phenotype	Ethnicity	N	Analyses	Center	Ethnicity	N	Analyses
Utah	Azoo/Oligo	Caucasian	83	C,A	Utah	Caucasian	62	C,A
Weill Cornell	Azoo	Caucasian	420	C,R,A	UKNBS	Caucasian	974	C
Porto	Azoo/Oligo	Caucasian	162	C,A	Spain	Caucasian	622	A
WUSTL	Azoo/Oligo	Caucasian	61	C,A	WUSTL	Caucasian	100	C,A
Nanjing	Azoo	Han Chinese	979	R	Nanjing	Han Chinese	1734	R

'N', number of individuals in the cohort after excluding ethnic outliers and samples with poor data quality. 'Analyses', describes whether the cohort was included in primary CNV analyses ('C'), replication CNV analyses ('R'), and autozygosity analyses ('A'). Note that due to small sample sizes, the 17 Weill Cornell samples with SNP array data were merged with Porto samples and the combined set treated as a single cohort for the primary CNV analyses. Thus the total number of cases with whole-genome array data are 83+17+162+61 = 323. Many more samples were sourced from Cornell for replication analysis. Full details of each cohort are available in Text S1.
doi:10.1371/journal.pgen.1003349.t001

found no single-locus associations, at these hotspot loci, or elsewhere, that met the strict criteria of genomewide significance in both the discovery and replication cohorts. Many of our single-cohort associations from one platform lack adequate probe coverage on other platforms for robust replication (Text S1). However, several loci were significant on joint analysis of all cohorts.

The best candidate for a novel locus generating NAHR-mediated infertility risk mutations is a 100 kb segment on chromosome Xp11.23 flanked by two nearly identical (>99.5% homology) 16 kb segmental duplications containing the sperm acrosome gene *SPACA5* (Figure 2a, Figure S7). We identified 9 deletions of this locus spread across all patient cohorts (3 in PT, 1 in UT, 5 in WUSTL) compared to 8 in the pooled 1124 controls (2.8% frequency versus 0.7%, odds ratio = 3.96, p = 0.005, Fisher exact test). We genotyped the deletion by +/− PCR in an additional cohort of 403 men with idiopathic NOA from Weill Cornell, and observed an additional 3 deletions (Figure S8, Text S1). In a prior case-control study of intellectual disability, investigators using qPCR estimated the allele frequency of this

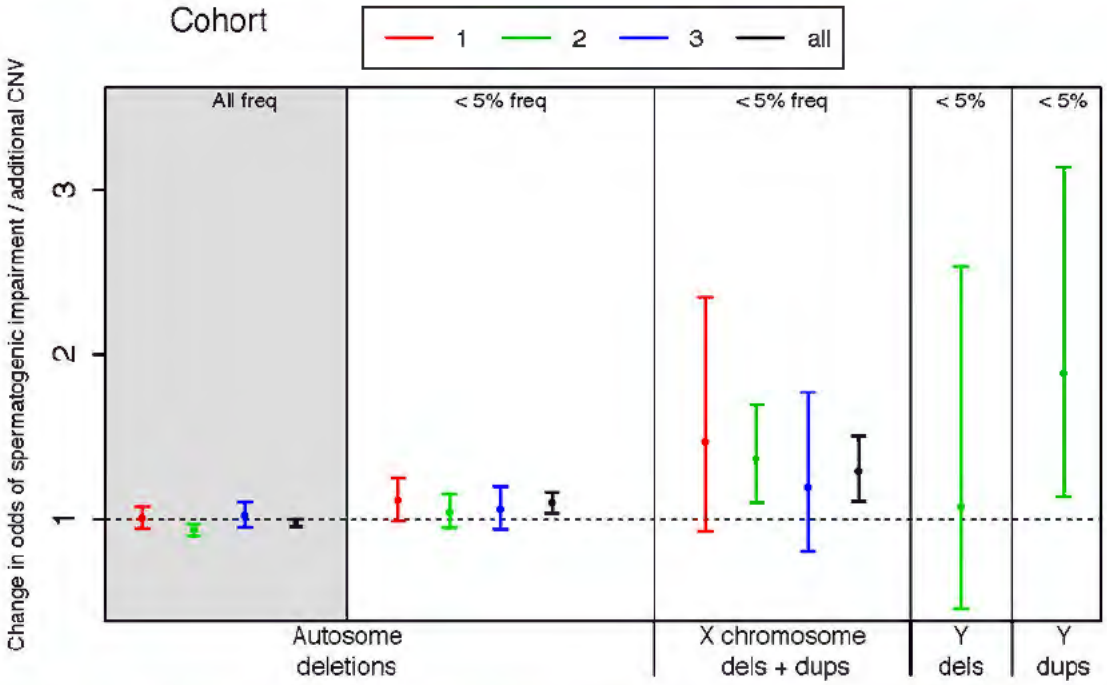

Figure 1. Rare variant burden in cases of spermatogenic impairment. We used logistic regression to estimate the influence of copy number variants (CNVs) on the odds of being diagnosed with impaired spermatogenesis in three case-control cohorts. The estimated odds of spermatogenic impairment is equal to, or slightly lower than, one when considering autosomal deletions of all frequencies (leftmost panel, shaded grey). However, when considering only autosomal deletions with call frequencies less than 5%, we observed a progressively increasing risk conferred by events on the autosomes, the X and the Y chromosomes. A very small number of Y-linked calls were made in cohorts 1 and 3 due to array design, thus we have only plotted Y-linked rates for cohort 2. Samples with Y-linked AZF deletions were excluded from the study. The odds ratio estimated from fitting a logistic regression model of total CNV count to disease status is plotted separately for each cohort, as well as the combined set of all cohorts (black points). Cohort 1 = Utah (Illumina 370K), 2 = Porto and Weill Cornell (Affymetrix 6.0), 3 = WUSTL (Illumina OmniExpress), All = meta-analysis of all three cohorts. Sample sizes used in CNV analysis are n = 83 cases and 62 controls for cohort 1, n = 183 cases and 974 controls for cohort 2, and n = 61 cases and 100 controls for cohort 3.
doi:10.1371/journal.pgen.1003349.g001

deletion to be 0.47% (10/2121) in a large Caucasian male control cohort [33]. Combining these data, we estimate the allele frequency of the deletion to be 1.6% in Caucasian cases, compared to 0.55% in Caucasian controls (OR 3.0, 95% CI 1.31–6.62, p = 0.007). The deleted region contains the X-linked cancer-testis (CT-X) antigen gene *SSX6*; the CT-X antigen family is a highly duplicated gene family on the X chromosome comprising 10% of all X-linked genes and is expressed specifically in testis. After controlling for differences in coverage across the array platforms used in this study, we find a significant enrichment of rare deletions of CT-X genes in all cases (p = 0.02); this finding did not extend to duplications or CT antigen genes on the autosomes (Table 2).

When analyzing all cohorts jointly, our strongest association (genomewide corrected p-value <0.002) is to both gains and losses involving a 200 kb tandem repeat on Yq11.22, *DYZ19* (Figure S6, Figure S9), a human-specific array of 125 bp repeats first discovered as a novel band of heterochromatin in the Y chromosome sequencing project [34]. Tandem repeat arrays are often highly unstable sequence elements that can mutate by both replication-based and recombination-based (e.g. NAHR) mechanisms. In our data there were 9 gains and 11 losses at *DYZ19* in 323 cases (combined frequency 6.1%), compared to 3 gains and 12 losses in 1136 controls (combined frequency 1.3%). While this finding may ultimately require painstaking technical work to conclusively validate, we have several reasons to believe the association is real. First, we have previously shown that it is possible to identify real copy number changes at VNTR loci using short oligonucleotide arrays [35]; second, copy number changes at this locus were identified by multiple platforms in the current study; third, the association is nominally significant in both the Utah and Porto cohorts; fourth the locus is within the AZFb/c region. The direction of copy number changes does appear to track with haplogroup – while 12/13 duplications occur on the R1 background, 14/15 deletions for which haplogroup could be determined occur on I or J background. Haplogroup assignments for the carriers of these CNVs were confirmed by standard short tandem repeat analysis (Text S1). The strong association between haplogroup and direction of copy number change is noteworthy; it may indicate that DYZ19 CNVs are merely correlated with other functional changes on these chromosomes, or perhaps the structure of these chromosomes predisposes them to recurrent gains (R1) or losses (I/J).

The gene *DMRT1* is widely believed to be the sex-determination factor in avians, analogous to *SRY* in therians, and may play the same or similar role in all species that are based upon the ZW sex chromosome system [36]. *DMRT1* encodes a transcription factor that can activate or repress target genes in Sertoli cells and premeiotic germ cells through sequence-specific binding [37]. In humans, *DMRT1* is located on 9p24.3 in a small cluster with the related genes *DMRT2* and *DMRT3*. Large terminal deletions of 9p are a known cause of syndromic XY sex-reversal, and although the role of the *DMRT* genes in the 9p deletion syndrome phenotype has not yet been defined, mouse experiments have shown that homozygous deletion of *DMRT1* causes severe testicular hypoplasia [38,39,40].

We found two, perhaps identical, 132 kb deletions spanning *DMRT1* in the Utah cohort in men with azoospermia, and a 1.8 Mb terminal duplication of 9p, spanning these genes, was seen in a single normozoospermic control from Utah (Figure 2b). All three of these rearrangements were validated by TaqMan assay (Figure S10, Text S1). Both men were recruited into the study in Salt Lake City, UT between 2002 and 2004. They self-reported their ancestry as Caucasian, and in both cases this assumption was clearly verified by principal components analysis of their genetic data (Figure S2). There was no evidence that the two deletion carriers were closely related upon comparison of their whole-genome SNP genotypes. Testis biopsies were performed on both men; these indicated apparent Sertoli cell only syndrome in the first and spermatocytic arrest in the second. Both men exhibited apparently normal male habitus and virilization with no phenotypic similarities to 9p deletion syndrome.

We obtained Affymetrix 6.0 array data from a previously published genomewide association study of idiopathic NOA in Han Chinese [41] comprised of 979 cases and 1734 controls (Text S1). After processing these samples with our CNV calling pipeline, we observed an additional 3 deletions of *DMRT1* exonic sequence in cases (0.3%) and none in controls (Figure 2B, Figure S11). From these combined array data we estimate a frequency of *DMRT1* exonic deletion of 0.38% (5/1306) in cases and 0% (0/2858) in controls (OR = Infinity, [2.0-Inf], p = 0.003). We obtained the two largest control SNP array datasets in the Database of Genomic Variants (DGV), representing CNV calls from 4519 samples typed with platforms of equal or higher probe density to the ones used here [42,43]. None of these samples contained CNV of any sort affecting *DMRT1*. Finally, we screened an additional set of 233 idiopathic NOA cases from Weill Cornell, and 135 controls with the TaqMan validation assay and identified an additional 3 deletions (2 in cases, 1 in controls, Text S1, Figure S12). As this qPCR assay interrogates intronic sequence, the functional consequences of these 3 deletions are unclear. Our array data have revealed some of the smallest coding deletions of *DMRT1* reported to date in humans, and should help to clarify the critical regions of 9p involved in testicular development and function.

Notably, using a bespoke reanalysis of the intensity data, we did not see evidence for CNVs involving the gene *PRDM9*, a recently characterized zinc finger methyltransferase that appears to control the location of recombination hotspots in a diversity of mammalian species. Heterozygosity of *PRDM9* zinc finger copy number has been shown to cause sterility in male hybrids of *Mus m. domesticus* and *Mus m. musculus* due to meiotic arrest [44].

Functional impact

The identification of functional or physical annotations enriched in case-associated CNVs can be a powerful step in constructing models to classify pathogenic variants. We searched for significant case-specific aggregation of CNVs in several classes of functional sequence, including 195 genes previously shown to result in spermatogenic defects when mutated in the mouse [45], all protein and non-protein coding genes, and 525 testis genes that are differentially expressed during human spermatogenesis (Text S1). Deletion of X- or Y-linked exonic sequence conferred the strongest risk (OR = 1.87 [1.30–2.68], $p<1\times10^{-3}$). Very similar risk was associated with deletion of exonic sequence from testis genes differentially expressed during spermatogenesis, despite the fact that only 15% of these genes are located on the sex chromosomes (OR = 1.85 [1.01–3.39], $p<0.05$). Deletion of any exonic sequence was also associated with disease (OR = 1.25 [1.07–1.46], $p<5\times10^{-3}$). Deletion of miRNAs was not associated, nor was deletion of the 195 mouse spermatogenic genes [45], which were very rarely deleted in either cases or controls.

We hypothesized that at least some of the functional impact of CNV burden on fertility was a result of disruption of haploinsufficient (HI) genes, as has been demonstrated for neuropsychiatric and developmental disease [46]. For each singleton deletion in our collections we used a recently described modeling framework to calculate the probability that the deletion is pathogenic due to

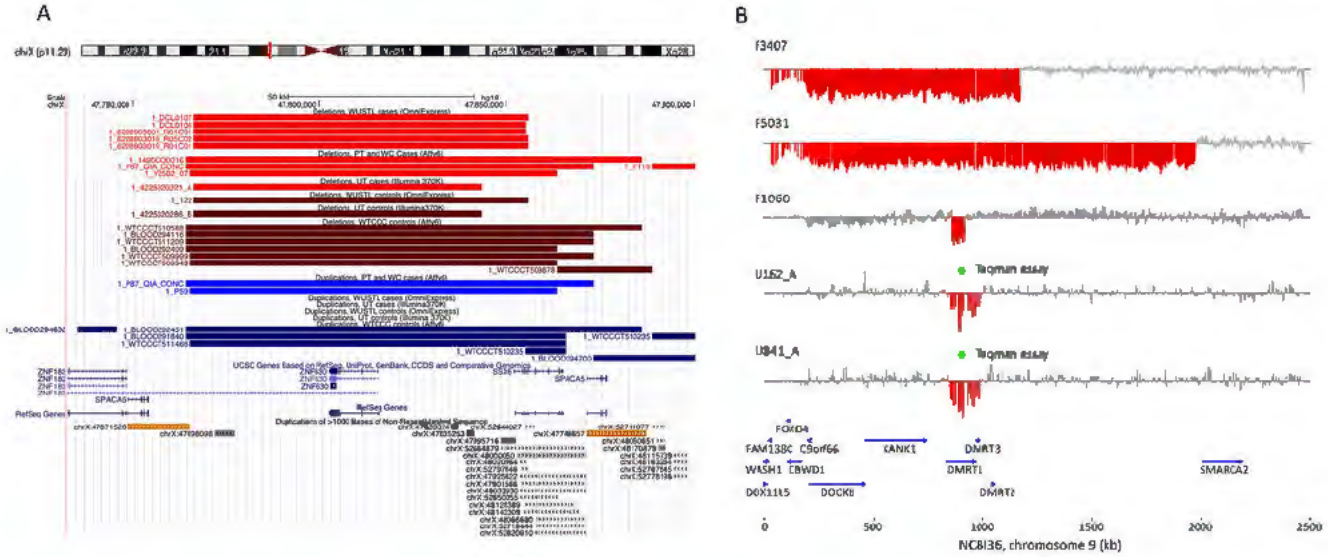

Figure 2. Discovery of recurrent deletions in azoospermia. (A) A recurrent microdeletion on Xp11.23 (47765109–47871527 bp, hg18) is a strong candidate risk factor for spermatogenic failure. The location of deletions (red shades) and duplications (blue shades) in cases and controls are plotted separately for each cohort. CNVs at this locus appear to arise due to non-allelic homologous recombination between two nearly identical (>99.5% homology) 16 kb segmental duplications that contain the sperm acrosome gene *SPACA5*. Also within the CNV region are the genes *ZNF630* and the cancer-testis antigen *SSX6*. We identified 9 deletions of this locus spread across all patient cohorts (3 in PT, 1 in UT, 5 in WUSTL) compared to 8 in the pooled 1124 controls (2.8% frequency versus 0.7%, odds ratio = 3.96, p = 0.005, Fisher exact test). After analysis of an additional 403 cases and 2121 controls, the association is still significant (combined data: 1.6% frequency in cases, 0.55% in controls, OR 3.0, 95% CI = [1.31–6.62], p = 0.007). (B) We identified two patients with deletion of *DMRT1*, a gene on 9p24.3 that is orthologous to the putative sex determination locus of the avian ZW chromosome system [36]. Both men were diagnosed as azoospermic. We validated these deletion calls with a qPCR assay (green star, Figure S9). We screened Affymetrix 6.0 data from an independent Han Chinese case-control study of NOA and identified an additional 3 deletions of *DMRT1* coding sequence in 979 cases and none in 1734 controls. Finally, we observed no coding deletions of *DMRT1* in the two largest control SNP array datasets in the Database of Genomic Variants, consisting of 4519 samples [42,43]. The combined results indicate that deletion of *DMRT1* is a highly penetrant genetic cause of human spermatogenic failure (frequency of 0.38% in 1306 cases and 0% in 7754 controls, combined $p = 6.2 \times 10^{-5}$). Patient IDs are indicated next to each plot (U162_A, U841_A = Utah cohort patients; F3407, F5031, F1060 = Nanjing cohort patients).
doi:10.1371/journal.pgen.1003349.g002

dominant disruption of a haploinsufficient gene [47]. Much to our surprise, HI scores from deletions in infertility cases were much smaller than those from cases of autism and developmental disorders and in fact indistinguishable from controls (mean HI score −1.16 in controls, −1.02 in all spermatogenic impairment cases, p = 0.49 by Wilcoxon rank sum test; Figure 3). Likewise there was no enrichment of large rearrangements within 45 known genomic disorder regions in cases [46]. In contrast to previously

Table 2. X-linked cancer-testis antigens deleted in case and control samples.

GENE	START**	STOP	PT/WC	UTAH	WUSTL	CASE COUNT	CONTROL COUNT
SSX6†	47852031	47865013	3	1	5	9	8
SSX1	47999740	48011823	0	1	0	1	2
SSX3	48090806	48101086	0	0	0	0	1
GAGE10	49047068	49063255	0	0	0	0	1
NXF2B	101501974	101613388	1	0	0	1	1
CT47*	119895375	119898693	1	1	0	2	1
CT45*	134674850	134684654	9	0	0	9	21
SPANXA1/A2*†	140499461	140500526	0	0	0	0	6
MAGEA11†	148575476	148604507	0	1	0	1	0
MAGEA9†	148671401	148677206	0	0	0	0	1
MAGEA8†	148770653	148775266	1	0	0	1	0
Unique Samples						24 (7.3%)	42 (3.7%)***

*Gene or gene family is annotated multiple times on the reference genome; coordinates for the first copy are given.
**Gene coordinates are based on NCBI36.
***Frequency difference between cases and controls, $p<0.05$.
†Patient-specific deletions of these genes were reported in a study of X-linked CNVs in over 250 azoospermia cases and 300 normospermic controls [58].
doi:10.1371/journal.pgen.1003349.t002

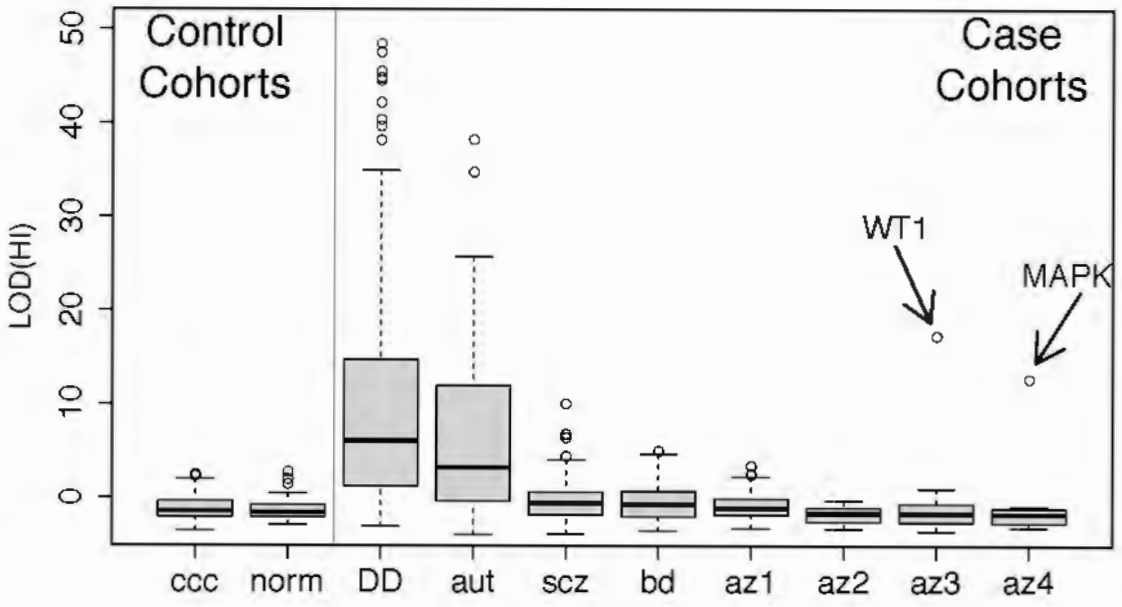

Figure 3. Disruption of predicted haploinsufficient genes is infrequent in spermatogenic failure. We obtained lists of rare deletions, left panel, from the Utah and WTCCC control cohorts and, right panel, from cohorts of developmental delay (DECIPHER) [66], autism [67], schizophrenia [68], bipolar disorder [66,68], and spermatogenic impairment (this study). We used a published method for assessing the likelihood that each deletion disrupts a haploinsufficient gene [47], summarized as a LOD score, and ordered each cohort by the median LOD(HI) within cases and controls separately. While the CNVS from DECIPHER ($p<1\times10^{-15}$), autism ($p<1\times10^{-15}$), schizophrenia ($p<1\times10^{-4}$) and bipolar disorder ($p<0.002$) show significant enrichment of high LOD (HI) scores compared to controls, the infertility cohorts have score distributions indistinguishable from controls. Two outlier deletions from the infertility cohort are annotated; one is a deletion of *WT1*, a key gene in gonadal differentiation, and the other is a 1 Mb deletion involving several genes including *MAPK1* and the cancer-testis antigen *PRAME*. Further review of clinical data from the *WT1* carrier showed signs of cryptorchidism. Abbreviation of azoospermia cohorts: az1, Utah cohort, az2, WUSTL, az3 Porto, az4, Weill-Cornell. Note that for additional detail we have split the cohort referred to as "Porto" in the main text into two subgroups, az3 and az4, defined by the clinical group that ascertained the cases.
doi:10.1371/journal.pgen.1003349.g003

described diseases that feature CNV burden, spermatogenic impairment may be more likely to result from large effect recessive mutations, or perhaps the additive effect of deleterious mutations across many loci. We sought to uncover support for recessive mutation load in our cases by assessing the impact of inbreeding, or elevated rates of homozygosity, on disease risk by applying a population genetic approach to the SNP genotype data from our samples [48].

HBD analyses

The major genetic side effect of consanguineous mating is a genome-wide increase in the probability that both paternal and maternal alleles are homozygous-by-descent. This probability is often summarized as the inbreeding coefficient, *F*, and can be estimated from analysis of pedigree structure or by direct observation of genomewide SNP genotypes.

Due to differences in demographic history and culture, the extent of background homozygosity in the genome is expected to vary when comparing diverse populations throughout the globe. The haplotype modeling algorithms implemented in the software package BEAGLE estimate the background patterns of linkage disequilibrium and homozygosity across a set of samples, allowing population-specific information to be used to assess the evidence that any given section of a genome is likely to be homozygous-by-descent (HBD). During the course of our study we concluded that standard PCA-based approaches to stratification are insufficient to correct for population structure during the analysis of inbreeding, even when using population genetic methods like BEAGLE (Text S1, Figure S13). The problem comes not from spurious identification of HBD, but from spurious association of HBD with disease status when case and controls are sampled from groups with different levels of background relatedness. For instance, in a recent survey of 17 Caucasian cohorts, estimates of the average inbreeding coefficient, *F*, varied from 0.09% to 0.61%, with UK-based cohorts showing the lowest *F* and the one Portuguese cohort showing the highest [27]. While PCA-based methods traditionally detect and correct for differences in allele frequencies among groups, we believe that they do not detect differences in inbreeding that can be readily incorporated into a case-control testing framework. In the following section, we use data from 622 healthy adults from Spain, who we believe form a more appropriate control group for the Porto case cohort (Methods, Text S1, Figure S13).

Analyzing each cohort separately, BEAGLE identified 5343 chromosome segments likely to represent HBD regions (HBDRs) across all samples. We excluded low-level admixture as a spurious source of HBD (Figure S3). Only three of these segments were identified as apparent artifacts induced by large heterozygous deletions (287 kb, 817 kb, and 877 kb in size) and were removed before subsequent analyses. As expected, the distribution of HBD across all samples was L-shaped, with the majority of HBDRs shorter than 1 Mb and a few intermediate and very large events observed (Figure 4b). The largest HBDR identified spanned all of chromosome 2 in an azoospermic individual, indicative of uniparental isodisomy of the entire chromosome. Clinical reports of UPD2 are extremely rare – there are 7 previous reports of UPD2 that have been ascertained through association with an autosomal recessive disorder [49]. In each of these cases a recessive disorder that lead to clinical presentation was identified. There is currently no proof of imprinted genes on chromosome 2 from

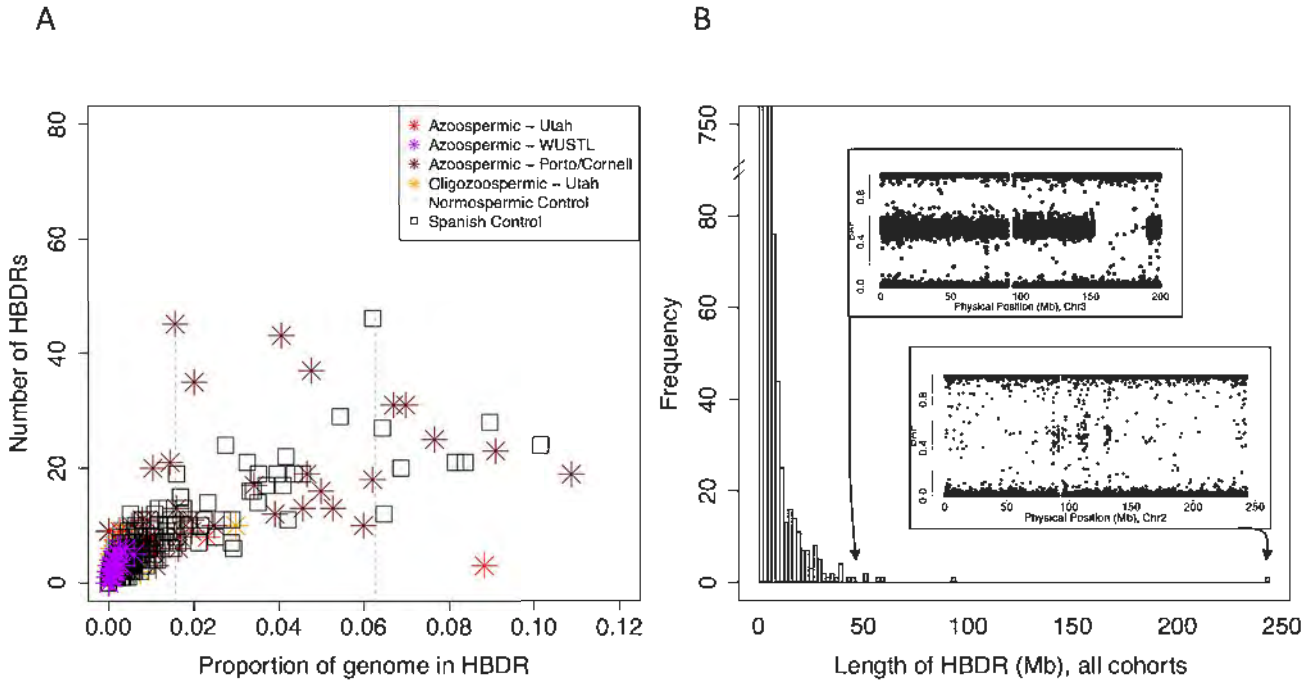

Figure 4. Patterns of homozygosity in men with low sperm count. (A) Distribution of the number of HBD regions (HBDRs), and the proportion of genome contained in these putative HBD regions, plotted for each sample in this study. Replication case and control cohorts are indicated in the legend. (B) Length distribution of HBDRs detected in all samples combined. Inset, two panels showing probe level intensity data corresponding to the two largest HBDRs detected. BAF: b-allele frequency, calculated as B/(A+B) where A and B are the approximate copy numbers for the A and B allele, respectively. The largest HBDR detected corresponds to a case of uniparental disomy of chromosome 2 (UPD2) detected in an azoospermic man from the Utah cohort.
doi:10.1371/journal.pgen.1003349.g004

either mouse or human data. We performed whole exome sequencing on this individual, and using a simple scoring scheme based on functional annotation and population genetic data, identified a homozygous missense mutation of the *INHBB* gene as the most unusual damaging homozygous lesion in the genome of this individual (Figure 5, Text S1). The biology of the *INHBB* gene product strongly implicates this mutation as a causal factor but without additional functional or epidemiological evidence such a conclusion is speculative (Figure 6).

Setting aside this case of UPD2, we found only modest evidence for an enrichment of homozygosity in men with spermatogenic impairment (Figure 4a, Table 3). Our hypothesis was that, if a large percentage of cases of azoospermia were attributable to large-effect autosomal recessive Mendelian mutations, we would see a corresponding increase in the proportion of cases with large values of *F*. The average inbreeding coefficient was numerically higher in each case cohort compared to its matched control cohort (Table 3). We used a logistic regression mixed model framework to test for association between autozygosity and disease, while controlling for population structure, fitting models that treated autozygosity as both a categorical variable (e.g. inbreeding coefficient >6.25%, yes or no) and a continuous variable (*F*, Methods). While the estimated effect of inbreeding on disease risk was positive in every model that we tested, the corresponding odds ratios did not differ significantly from 1 in any version (Table 3). There were fewer than 10 HBD regions shared by 2 or more cases, supporting the model that spermatogenic efficiency has a polygenic basis. We also tested for case-specific aggregation of HBD segments using the same association framework as that used for CNVs. We did not identify any significant patterns. Based on published analyses of small-effect recessive risk mutations in other complex diseases, we believe our current sample size would be underpowered to detect association between very old inbreeding (e.g. due to shared ancestors 15 generations ago). It is possible that large cohorts, consisting of over 10,000 cases, may be needed to accurately estimate the relationship between low-level variation in inbreeding (*F* values smaller than 0.1) and azoospermia risk, as well as map specific risk alleles [27,50].

Discussion

We report here the largest whole genome study to date investigating the role of rare variants in infertility, examining data from 323 cases of male infertility and 1,136 controls. These data demonstrate that rare CNVs are a major risk factor for spermatogenic impairment, and while confirming the central role of the Y chromosome in modulating spermatogenic output, our risk estimates for autosomal and X-linked CNVs indicate that this phenotype is influenced by rare variation across the entire genome. The controls from two of the cohorts were unphenotyped, and given the estimated prevalence of azoospermia (1%), we may have underestimated the risk associated with these large rearrangements.

We observed 5 deletions of *DMRT1* coding sequence in cases and none in over 7,000 controls. These deletions ranged in size from 54 kb to over 2 Mb (Table 4). *DMRT1* is situated in a region of chromosome 9p that has been identified as a source of syndromic and non-syndromic forms of XY gonadal dysgenesis (GD). The deletions of this region that are associated with syndromic forms of GD are usually 4–10 Mb in size, while isolated GD has been reported for deletions smaller than 1 Mb [40,51,52]. Despite frequent involvement of *DMRT1* in these putative causal mutations, there is variability in both the phenotypic outcome

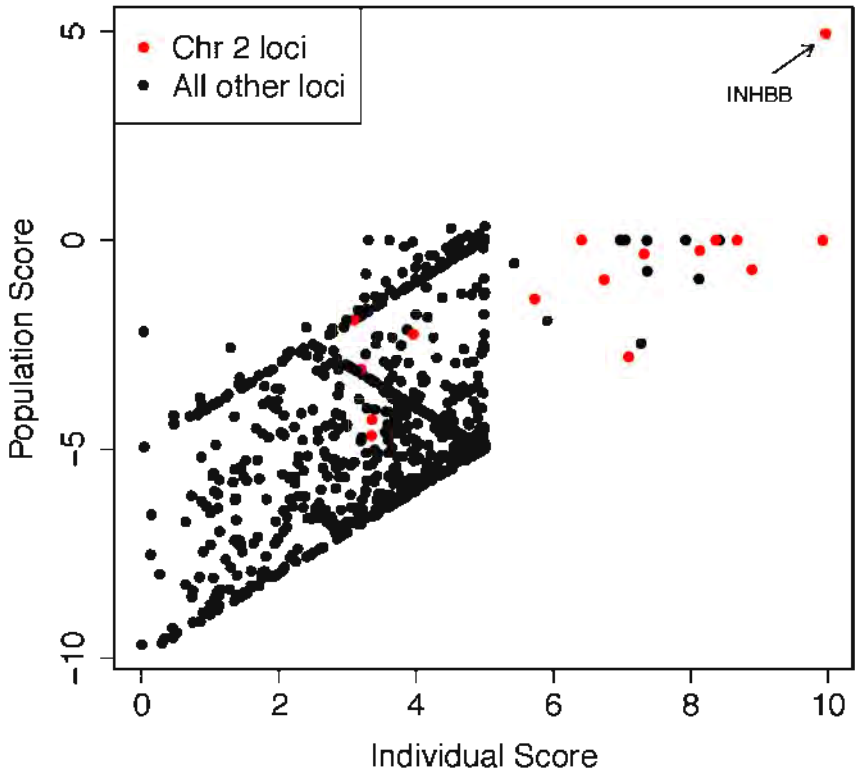

Figure 5. Analysis of exome sequencing data identifies a candidate azoospermia mutation in the case of UPD2. We performed whole-exome sequencing on the case of UPD2 in an attempt to identify a potential genetic cause for this man's azoospermia. We constructed a scoring method to rank order the exome variants in two dimensions: (i) within the set of variants seen in this single exome, the "Individual Score" and (ii) across a large set of exome sequences, the "Population Score". For each exome variant, the Individual Score, $P_{ind,}$, was constructed by summing normalized predictions of functional impact from 5 commonly used annotation algorithms: PhyloP, PolyPhen2, SIFT, GERP, and LRT. This score was then multiplied by the ploidy of the mutant allele (e.g. 1× for a heterozygous genotype and 2× for a homozygous genotype) creating a final Individual Score ranging from 0–10. We also calculated the Individual Score for all variation in the 1000 genomes Phase I sequencing data. To construct the "Population Score" for each variant in the UPD individual, P_{pop}, we identified the maximum Individual Score variant in the corresponding gene, P_{max}, within the 1000 genomes data, and defined $P_{pop} = P_{ind} - P_{max}$. The purpose of the Population Score is to scale the importance of each Individual Score by the extent of pathogenic variation that exists in the population at each gene. Only sites with minor allele frequencies less than 10% in both the 1000 genomes data and the Exome Variant Server (http://evs.gs.washington.edu/EVS/) were considered in the analysis. When examining the joint distribution of P_{pop} and P_{ind} for the UPD2 individual, we saw an enrichment of large scores for variants on chromosome 2, as expected. The most extreme variant on both scales was a homozygous nonsense mutation in the gene *INHBB*, the implications of which we discuss in Figure 6.
doi:10.1371/journal.pgen.1003349.g005

affiliated with each deletion and the extent of *DMRT1* coding sequence contained therein. At least two cases of GD have been linked to deletions near but not overlapping *DMRT1* – one 700 kb mutation 30 kb distal to *DMRT1* in a case of complete XY GD that was inherited from an apparently normal mother, and a second 260 kb *de novo* deletion about 250 kb distal to *DMRT1* [39,40]. Both of these deletions overlapped the genes *KANK1* and *DOCK8*. On the other hand, two smaller deletions, one a 25 kb deletion of *DMRT1* exons 1 and 2, and one a 35 kb deletion of exons 3 and 4, have been observed in patients with complete GD and bilateral ovotesticular disorder of sexual development, respectively [51,52]. Based on the clinical records of patients in our current study, there is no chance that our *DMRT1* deletion carriers could represent misdiagnosis of a condition as severe as complete XY GD, which presents with the appearance of female genitalia. Indeed, two of our *DMRT1* deletion carriers were subject to testicular biopsies. Our observations here suggest that hemizygous deletion of *DMRT1* is a lesion that shows variable expressivity that may depend on the sequence of the undeleted *DMRT1* allele, variation in other sequences on chromosome 9p, and the state of other factors in the pathways regulating testicular development and function. Strictly speaking, statements that hemizygous deletions of *DMRT1* are "sufficient" to cause GD or spermatogenic failure need to be qualified at this point until we gain a better understanding of the effects of genetic background. For instance, in most studies of *DMRT1* deletion, the undeleted *DMRT1* allele is rarely sequenced. Is the mode of action dominant or recessive?

Deletions of the Y chromosome have long been appreciated as a cause of azoospermia, and we have now shown here that Y-linked duplications are also significant risk factors for spermatogenic failure. The precise definition of the duplication sensitive sequences awaits further investigation. Historically, Y duplications have been much less studied than Y deletions, as +/− STS PCR is the standard assay for assessing Y chromosome copy number variation in both the clinical and research setting. Quantitative PCR methods for measuring Y chromosome gene dosage have been described in the literature, and applied almost exclusively to studying the phenotypic effects of duplication of genes in the AZFc region [53]. Results of these investigations are conflicting, with studies of Europeans reporting no association between AZFc partial duplication and spermatogenic impairment [54], while

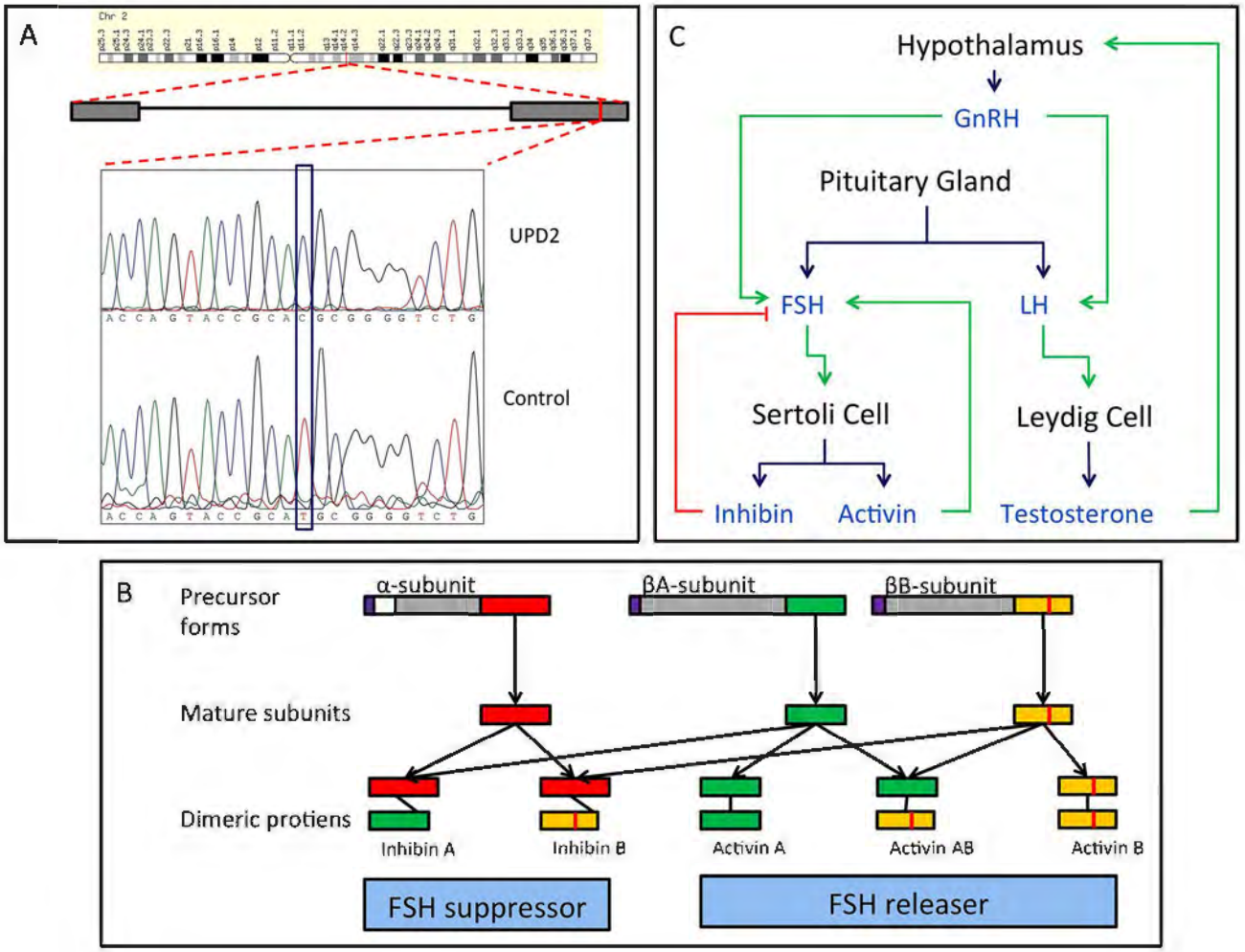

Figure 6. Homozygous missense mutation of *INHBB* identified in the case of UPD2. (A) We validated this candidate by Sanger sequencing in the UPD2 case and control individuals. Mutant and reference nucleotides are highlighted within the blue box, confirming the homozygous T to C nucleotide change observed at chr2:12,1107,305 bp (hg19) of the UPD2 individual. Grey boxes represent the exons of the gene and the red line indicates the location of the observed mutation within the gene. (B) *INHBB* encodes for the protein, Inhibin βB, which along with inhibin α and inhibin βA, combine combinatorially to form the inhibins and activins. Each protein expressed by *INHA*, *INHBA*, *INHBB* consists of an N-terminal signal peptide (purple), a propeptide (grey), and a subunit chain (green, red or yellow). The mutation identified here results in a M370T change of the inhibin βB subunit chain (location indicated by a vertical red line throughout the diagram). The various inhibin subunits dimerize via disulfide bonds (locations indicated by black lines between subunits). As the βB subunit participates in multiple complexes with antagonistic functions, the functional consequences of loss-of-function or gain-of-function mutations in this protein may be difficult to predict. (C) The role of inhibins and activins in the hypothalamic-pituitary testicular axis. These complexes have diverse functions in the body, but are most well known for their ability to stimulate and inhibit follicle stimulating hormone (FSH) production, a process critical for spermatogenesis. Blue arrows connect hormones to the cell or gland by which they are secreted. Green arrows indicate stimulatory interactions, and red lines indicate inhibitory interactions.
doi:10.1371/journal.pgen.1003349.g006

reproducible associations have been reported in east Asian cohorts [55,56]. Notably, we identified some duplications on the Y chromosome greater than 2.5 Mb in size, all spanning the AZFc locus (Figure S6), in 8/179 cases (those typed on Affymetrix 6.0), compared to 13/972 controls (OR 3.45 [1.21–9.12], $p<0.01$). Rearrangements of this size on the autosomes confer staggering risk for other forms of disease; for example, by one recent estimate CNVs larger than 3 Mb have an OR of 47.7 for intellectual disability and/or developmental delay [46]. Our results suggest that Y chromosome structure may be more dosage sensitive than previously appreciated, and we speculate that some genes and non-coding sequences of the Y chromosome may be under stabilizing selection for copy number [57].

Three recent studies have used array-based approaches to characterize CNVs in men with azoospermia. Our finding of an X-linked CNV burden in men with spermatogenic failure has been replicated and described elsewhere [58]. In a second study, Tuttelmann *et al.* evaluated 89 severe oligozoospermic, 37 azoospermic, and 100 normozoospermic control men using Agilent 244K and 400K arrays and identified a number of CNVs potentially involved in male infertility [24]. Third, Stouffs *et al.* assayed nine azoospermic men and twenty control samples using the 244K array and followed-up CNVs of interest by q-PCR in up to 130 additional controls [25]. Using the criterion of at least 51% reciprocal overlap, we have identified a number of CNVs in the current study that overlap with case-specific CNVs in the Tuttelmann and Stouffs studies. The majority of these CNVs appear to be relatively common polymorphisms and not case-specific in our larger dataset; however several noteworthy CNVs overlap between studies and are absent, or are present at a very

Table 3. Summary of inbreeding coefficient estimates across cohorts, and association testing.

			F>0.5%	*F*>1.6%	*F*>6.25%	All *F*
Cohort	**Type**	**Average *F***	**# samples**	**# samples**	**# samples**	**# samples**
Porto	Case	0.0069	39	21	5	175
Spain	Control	0.0042	112	41	8	622
Utah	Case	0.0020	5	3	1	84
Utah	Control	0.0014	0	0	0	59
WUSTL	Case	0.0027	6	0	0	70
WUSTL	Control	0.0020	1	0	0	99
Effect			OR 1.25 (95% CI= [0.81–1.92])	OR 1.62 (95% CI= [0.88–2.98])	OR 1.18 (95% CI= [0.34–4.03])	β=8.23 (95% CI= [1.92–14.54])
p value			0.31	0.12	0.794	0.19

For each case and control group we present the average the estimated inbreeding coefficient and the number of individuals with inbreeding coefficients above a specified threshold. The last column indicates the total number of individuals in each group. The bottom two rows indicate the results of an association test between inbreeding and case/control status using either a categorical variable as a definition of inbreeding status (*F*>0.5%, *F*>1.6%, and *F*>6.25%) or using the inbreeding coefficient as a continuous variable ("All *F*").
doi:10.1371/journal.pgen.1003349.t003

low frequency in controls. For example, Tuttelmann *et al.* identified a private duplication on Xq22.2 in an oligozoospermic man [24], and we identified an overlapping duplication in an oligozoospermic man from the present study (ChrX:103065826–103205985, NCBI36). These duplications alter the copy number of a small number of testis-specific or testis-expressed variants of histone 2B (H2BFWT, H2BFXP, H2BFM). No CNVs in this region were identified in more than 1600 controls. Tuttelmann *et al.* also identified an azoospermic man with a deletion and another with a duplication on 8q24.3, encompassing the genes *PLEC1* and *MIR661* [24]. We identified an oligozoospermic man with a duplication of the same region, affecting the same functional elements (chr8:145064091–145118650, NCBI36). CNVs of this locus are very rare, with a frequency of about 0.005% in our controls and 0.0025% in controls used for a recent study of developmental delay [46]. It is important to note that new variants will frequently be discovered whenever a discovery technology such as array CGH is applied to a new sample set, and the observation that a variant is patient-specific is not in itself remarkable, especially when one is investigating very small sample sizes.

Our observation of low deletion HI scores in cases raises a number of considerations for future studies of the genetics of spermatogenic impairment. We interpret low HI scores in cases as evidence against a widespread role for dominant, highly penetrant deletions in spermatogenic failure. It is possible that our case recruitment, which pre-screened for normal karyotype, may have removed all large HI score events; however our identification of two large HI deletions of *WT1* and *MAPK1* indicate otherwise (Figure 3). A second concern is that the data used to train the haploinsufficiency prediction algorithm is in part based on features of deletions known to cause dominant pediatric disease, and that an analogous approach trained on fertility phenotypes may lead to different conclusions. There are few examples of dominant loss-of-function mutations causing isolated infertility in humans and only 5 of the >200 mouse infertility mutants described in a previous review showed a phenotype in heterozygous form [45], so fitting a model of a dominant infertility mutation may be challenging in the short term. Nonetheless, developing disease-specific pathogenicity scores for infertility phenotypes should be a priority.

Table 4. *DMRT1* deletions detected by array in the current study.

Case	Start (bp)	End (bp)	Platform	DMRT1 Exons	Phenotype
U841_A	845091	994958	Illumina 370K	3,4,5	Azoo - SCOS
U162_A	853635	994958	Illumina 370K	3,4,5	Azoo - MA
F1060	861888	916779	Affymetrix 6	3,4	Azoo
F5031	30911	1972069	Affymetrix 6	All	Azoo
F3407	30911	1170987	Affymetrix 6	All	Azoo

'DMRT1 Exons' – exons contained within each deletion, numbered from the 5' to 3' position. SCOS – Sertoli Cell Only Syndrome; MA – maturation arrest. Deletion coordinates given with respect to NCBI36.
doi:10.1371/journal.pgen.1003349.t004

Despite the differences between the genetic signatures of spermatogenic impairment and severe developmental disease noted above, there are connections in their epidemiology. Recent results estimate a 9.9% rate of birth defects in children conceived by intracytoplasmic sperm injection (ICSI), the technology typically employed for assisting cases of severe male factor infertility, which is an OR of 1.77 compared to unassisted reproduction [59]. Among several possible explanations for this finding, our data raise the possibility that mutations that compromise gonadal function may act pleiotropically to disrupt development in other tissues. A better understanding of the genetic basis of male infertility is urgently needed in order to improve risk assessment for couples considering assisted reproduction.

Clinical genomics is a paradigm in need of robust applications, and our finding of a large CNV burden in cases suggest that some infertility mutations may have the high penetrance required for clinical utility. Indeed some mutation screens are already used clinically in the management of male infertility. Although the presence of azoospermia can be easily assessed using a standard laboratory test, many men with azoospermia will have sperm production within the testis and be candidates for testicular sperm retrieval. We have already identified that the specific AZF deletion (a, b or b/c) has a dramatic effect on the prognosis of sperm

retrieval (vs. AZFc-deleted males) [60]. In the present study, we have identified deletion of *DMRT1* coding sequence as a genetic event that appears highly predictive of spermatogenic failure. In depth characterization of carriers is now needed to understand how this mutation affects the prognosis of sperm retrieval. Similar whole genome tests may provide critical prognostic information that can help to characterize the chance of successful treatment for couples with non-obstructive azoospermia, avoiding expensive and needlessly invasive interventions, while potentially providing guidance for new therapeutic interventions.

Methods

Ethics statement

All DNA samples used in this study were derived from peripheral blood lymphocytes collected from individuals giving IRB-approved informed consent. The following IRBs were involved: INSA Ethics Committee and Hospital Authority (Portugal), University of Utah IRB, and Washington University in St. Louis IRB (#201107177). All samples of genomic DNA to be analysed in this study i) belong to DNA banks that have been established throughout the years; ii) are coded; and iii) each individual has signed a declaration of informed consent before donating his genomic DNA for analysis, authorizing molecular studies to be performed with this material.

Patient cohorts

All cases were deemed idiopathic following a standard clinical workup, which included screening for Y chromosome deletions. Controls from the Utah cohort were men with normal semen analysis, remaining controls were not phenotyped on semen quality. Full details of the source and diagnosis of samples in this study are available in Supplemental Methods. When using SNP arrays, CNV analysis is more sensitive to experimental noise than SNP genotyping, and we used different sample QC metrics to inform CNV and SNP stages of our project. As a result, we have slightly larger sample sizes for the HBD analyses than for the CNV analyses.

Population structure

The individuals studied here were sourced from diverse geographic locations (Table 1, Text S1). All primary samples (e.g. 323 cases and 1133 control samples subjected to whole-genome genetic analysis) were of self-reported Caucasian ancestry, but it was necessary to take additional steps to control for population structure in all aspects of the analysis. First, genetic ancestry of each sample was assessed by principal components analysis and ethnicity outliers were removed (Figure S2, Figure S3). Second, eigenvectors generated by this principal components analysis were used as covariates in both CNV association and inbreeding coefficient association analyses. For analyses focusing on the Y chromosome, we performed analyses conditioning on Y haplogroup to provide the most stringent possible correction for population structure with available data. Lastly, we conducted alternate association analyses with the Porto case cohort using a smaller, but more geographically proximal Spanish control cohort (Figure S5).

Identification of CNVs, regions of homozygosity-by-descent

Three array platforms were used for CNV discovery: Illumina 370K (Utah), Illumina OmniExpress (Washington University), and Affymetrix 6.0 (Porto, Cornell, Nanjing). Full details of sample processing and array experiments are available in Supplemental Methods. Three CNV calling algorithms were used to generate CNV maps for each individual typed with Illumina technology: GADA, a sparse Bayesian learning approach [61]; PennCNV, a Hidden Markov Model (HMM)-based method originally designed for the Illumina platform [62]; and QuantiSNP 2.0, another HMM-based method for Illumina [63]. CNVs called by 2 of 3 algorithms were retained for analysis. CNV calling for Affymetrix 6.0 was performed with Birdsuite [64]. Due to the complexity of calling CNVs on the sex chromosomes, for all array datasets we implemented a bespoke normalization and calling procedure that used only the GADA algorithm to call CNVs from the X and Y chromosomes. For full details of CNV calling see Supplemental Methods.

Regions of homozygosity-by-descent (HBD) were identified using BEAGLE 3.0 [48]. SNPs with no-call rates >5% were removed prior to HBD analysis. As BEAGLE uses a model for background linkage disequilibrium that is fit from the data, cases and controls from each cohort were analyzed simultaneously and separately to assess cohort-specific biases in calling HBD. Prior to downstream analysis, we identified and removed a small number of reported HBD regions that corresponded to rare, large hemizygous deletions.

Inbreeding coefficients for each individual were calculated from their HBD data using the formula:

$$F = (\text{total bases in HBDRs})/$$

$$\left(2.77 \times 10^9 \text{ total base pairs in SNP mappable genome}\right).$$

CNV and HBD association analyses

Due to differences in array content, CNV frequencies were determined on a per-platform basis. All CNV calls made on a given platform, in both cases and controls, were combined into CNV regions using a threshold of 50% reciprocal overlap to defined two events as the same ([35]). We defined the CNV frequency as the proportion of all samples (cases and controls) containing that CNV.

We constructed several statistical tests to measure differences between cases and controls. We used Mann-Whitney U tests to test for differences in the total amount of aneuploid sequence per genome. We used standard logistic regression to test for CNV load on chromosome compartments (e.g. the autosomes, X chromosome) and a small number of functional features (genes, miRNA, etc). To control for population structure these models included the first 10 principal components from PCA analysis of the SNP genotype data from all cohorts (Figure S2). We used a permutation strategy for genomewide, locus-by-locus testing for association at all genes and in 500 kb non-overlapping genomic windows. The permutation strategy, implemented with the software package PLINK, calculates nominal and genomewide p-values by permuting case-control labels [65]. To present consistent summaries of CNV burden for the entire study (all cohorts combined), we used linear mixed-effects logistic regression, treating cohort as a random factor and compared these to effect size estimates for each cohort separately using standard logistic regression (Figure 1). The mixed effects modeling framework controls for SNP platform as each case-control cohort was typed on a different platform; a similar use of mixed-effect modeling was recently described in a meta-analysis of schizophrenia SNP data [27].

Analogous tests were conducted on HBD segments from the original discovery cohort and the combined primary and replication datasets.

Validation assay

We performed validation and replication analyses of *DMRT1* deletions with and assay based on Taqman PCR. Copy number was assessed using a pre-designed assay #Hs06833797_cn within the *DMRT1* gene against an RNase P reference (assay # 4403326; both assays from Applied Biosystems, Carlsbad, CA, USA) according to manufacturer's recommendations.

Supporting Information

Dataset S1 Images of the normalized intensity data for all CNV calls in the Utah case-control cohort >100 kb in size. For each CNV, we have plotted the Log R Ratio (vertical lines) and B Allele Frequency (black points) of all probes within the CNV, as well as an equal number of probes 5′ and 3′ to the edges of the CNV. The Log R Ratio for probes within "gain" CNV calls are colored green, within "loss" CNV calls are colored red, and outside of a CNV call are colored grey. The sample ID and number of probes in the CNV call are listed above each image.
(PDF)

Figure S1 QC of Affymetrix callsets. Summary plots of array QC for the case samples and NBS control samples. There is an expected inverse correlation between the noise in the data (measured by the median absolute deviation (MAD) of the probe intensities) and the number of calls made in a particular experiment. We fit a linear model to these parameters separately for cases (A) and NBS controls (C), and samples >4 MADs from the fitted model were removed (circled dots). We also implemented an analogous QC step using the ratio of deletions/duplication calls per sample and number of calls per sample, separately for cases (B) and NBS controls (D), . In all plots, arrays are colored by their spatial autocorrelation function (a measure of "waviness"). Distributions of post-QC statistics are highly similar between cases and controls.
(TIF)

Figure S2 Principal components analysis (PCA) of population structure in all case cohorts post-QC. For each cohort, samples were analyzed together with HapMap samples using the EIGENSOFT package [69]. (A) Utah (B) WUSTL batch 1, (C) WUSTL batch 2, (D) Porto. Eigenvector loadings for cases and controls (A,B,C) or cases (D) are plotted as red crosses, while HapMap samples are plotted as other colored symbols described in each legend.
(TIF)

Figure S3 Analysis of population structure in the Porto cohort after sample QC. Based on the results of PCA analysis in Figure S2D, which indicate that the Portuguese population may have subtle differences in allele frequencies from northern European populations, we further investigated the possibility of population structure as a confounder. No significant correlation was observed between the estimated amount of African ancestry in each Porto case and (A) the total number of deletion calls or (B) the total number of rare deletions (here defined as <5% frequency). In both cases smaller (more negative) eigenvector loadings (x-axis) indicate a larger degree of African admixture. We segmented the genome of each sample into regions with 0, 1 or 2 chromosomes of African ancestry using the program HapMix [70]. (C) The percent ancestry inferred by HapMix in this way correlated well with PCA based ancestry estimates. (D) The extent of African ancestry in each case, as estimated by EIGENSTRAT, was uncorrelated (R = 0.002) with the fraction of the genome contained in a homozygous-by-descent region (a rough measure of the inbreeding coefficient), indicating that variation in distant African ancestry was not a major confounder of the HBD analyses.
(TIF)

Figure S4 Analysis of batch effects in Porto cases. The Porto arrays were run over a period of several months. Here we plot the total number of CNV calls per array, as a function of run order (sample number 1 = first array run, sample number 162 = last array run). Each dot is colored based on run date. No obvious outlier batches are visible. There was a small but insignificant trend for fewer CNV calls on later run dates (least-squares regression line is plotted).
(TIF)

Figure S5 CNV burden statistics using a Spanish control cohort. In the primary CNV analyses described in the main text, we use a Caucasian population from the United Kingdom as a control group for the Porto azoospermia cohort. Here, we address the effect of using a control group that is more closely matched on genetic ancestry. We performed the same burden analyses depicted in Figure 1 of the main text, this time using a much smaller control cohort of 368 Caucasian men ascertained in Spain. We used logistic regression to estimate the influence of copy number variants (CNVs) on the odds of being diagnosed with impaired spermatogenesis in three case-control cohorts. Eigenvectors from a principal components analysis were used as covariates as before. The odds ratio estimated from fitting a logistic regression model of total CNV count to disease status is plotted separately for each cohort, as well as the combined set of all cohorts (black points). Cohort 1 = Utah (Illumina 370K), 2 = Porto and Weill Cornell (Affymetrix 6.0), 3 = WUSTL (Illumina OmniExpress). Sample sizes used in CNV analysis are n = 83 cases and n = 62 controls for cohort 1, n = 179 cases and 368 controls for cohort 2, and n = 61 cases and 100 controls for cohort 3. Conclusion: While the direction of burden effect for rare autosomal deletions, X-linked deletions, and Y duplications was the same as seen with the analysis using UK controls, only the rare autosomal deletion burden model shows statistical evidence for an odds ratio greater than 1.
(TIF)

Figure S6 CNVs on the Y chromosome. (A) Our strongest statistical association involved gains and losses of a 200 kb tandem repeat termed *DYZ19*, approximately 500 kb distal to palindrome P4. Here are plotted 6 deletions from the UT cohort, which were evenly distributed between azoospermic and oligozoospermic men. (B) In the Weill-Cornell cohort, a small group of azoospermic individuals ascertained at a tertiary care clinic, we identified a number of classical AZF deletions, as well as duplications of AZFc. Next to each CNV is listed the sample ID and Y haplogroup of the sample inferred from SNP data (Methods). These data demonstrate that existing SNP platforms can cleanly identify Y chromosome rearrangements involving both gain and loss of sequence, and will facilitate investigation of the full spectrum of Y chromosome variation in future studies of male infertility. Notably, we observed complex patterns of copy number change in some samples that highlight the challenge of interpreting array data mapped to a single reference Y chromosome (haplogroup R1). In both panels, for each individual, deviations of probe $\log_2$ ratios from 0 are depicted by grey lines or black dots, and probes spanning CNV calls are colored as either red (losses) or green (gains).
(TIF)

Figure S7 CNV calls made in all array datasets at the Xp11 rearrangement hotspot. This plot is the same as Figure 2a, with the addition of tracks containing deletion and duplication calls from a

Spanish male control cohort assayed on the Affymetrix 6.0 platform.
(TIF)

Figure S8 Left, example STS PCR validation of Xp11 in one case from Cornell (F10) and two controls. Right, the same assay, run in multiplex (Xp11 and B-globin reactions in the same tube) for 5 WUSTL case carriers and two controls. Note the presence of the smaller beta-globin band in the two control individuals in lanes 7 and 8. The primer sequences for the Xp11 deletion assay and a control locus are given in the Text S1.
(TIF)

Figure S9 CNV calls made at the DYZ19 tandem repeat locus. CNV calls made on the Utah, Porto, and WUSTL case cohorts, and the Utah, NBS (WTCCC), WUSTL and Spanish control cohorts.
(TIF)

Figure S10 qPCR validation of DMRT1 deletions in the Utah Cohort. (A) Histogram of mean probe intensities from Illumina 370K array spanning the DMRT1/DMRT3 deletion locus (chr9:845901–994958 bp). N = 148 samples are plotted. (B) Taqman validation results for the *DMRT1* locus from 30 of the samples screened by 370K array in panel A (y-axis), including the two deletion carriers (red points) and one duplication carrier (green point) identified from Illumina 370K intensity data (x-axis).
(TIF)

Figure S11 CNVs on chromosome 9p in the Nanjing cohort. Affymetrix 6.0 data was generated on a cohort of 979 idiopathic NOA cases and ethnicity-matched controls 1734 controls recruited primarily from the cities of Nanjing and Wuhan, China. CNVs were called using the identical pipeline as the other cohorts. Plotted above are all of the deletions (red) and duplications (green) observed in these cases (top) and controls (bottom).
(TIF)

Figure S12 Detection of additional intronic *DMRT1* deletions using the TaqMan validation assay. As described in the supplemental methods, we screened 5 plates of DNA from Weill Cornell cases and 2 plates of Caucasian male controls using the *DMRT1* TaqMan validation assay. Each sample was assayed in quadruplicate (although some samples were assayed in duplicate or triplicate if insufficient DNA was available). We applied extremely stringent calling criteria to these data, excluding samples with (1) low DNA content as defined by picogreen assay (2) high standard deviation (>0.3) of delta CT measurements across replicates (3) low copy number confidence scores generated by CopyCaller software. Each point in the panels above represents the average delta CT for the control locus (VIC) and *DMRT1* (FAM) for a single sample. Red dots indicate deletion carrier calls. We detected 2 deletions in 233 case samples, a frequency of 0.86%, and 1 deletion in 135 controls (0.74%). As this assay is targeting intronic sequence, and we have not cloned the breakpoints the functional consequences of each deletion is unclear.
(TIF)

Figure S13 Controlling for population structure while testing for association between inbreeding and infertility. As described in Figure S2 and in Text S1, we used principal components analysis to assess population structure in our case and control cohorts. We use the BEAGLE software package to define homozygous-by-descent regions (HBDRs) of each sample in our study, and used these HBDRs to estimate corresponding inbreeding coefficients. We tested for association between inbreeding coefficient and the probability of azoospermia using a linear model. On the left, we show that there is strong association when analyzing data from the Porto cases and NBS Wellcome Trust case-control consortium controls (CCC controls) in a simple model that does not account for population structure ("No EV"). When including the first 10 eigenvectors from a PCA analysis ("EV"), the point estimate of association remains somewhat inflated but is no longer significant. On the right, we show the same analysis performed on the Porto cohort with a more closely matched control population from Spain, with clearly smaller confidence intervals and smaller point estimates of effect size. In order to be as conservative as possible, we report results of inbreeding analysis using the Spanish control cohort in the main text.
(TIF)

Table S1 The results of CNV burden tests performed on the Utah cohort. Each subtable contains summary statistics for a different subset of CNVs, selected by size and/or frequency.
(XLSX)

Table S2 The results of CNV burden tests performed on the Porto cohort. Each subtable contains summary statistics for a different subset of CNVs, selected by size and/or frequency.
(XLSX)

Table S3 The results of CNV burden tests performed on the Washington University cohort. Each subtable contains summary statistics for a different subset of CNVs, selected by size and/or frequency.
(XLSX)

Text S1 A description of patient cohorts, supplementary methods and analyses.
(DOCX)

Acknowledgments

We thank Luke Jostins for providing us with the code for the Yfitter haplogrouping algorithm and Dr. Gulum Kosova for assistance with sample preparation. We thank Dr. Graça Pinto and Sónia Correia, Unidade de Medicina da Reprodução, Maternidade Dr. Alfredo da Costa and Unidade Pluridisciplinar de Reprodução Humana, Hospital de SantaMaria Lisbon for the clinical evaluation of the patients. Thanks to Dr. J. Brendan M. Mullen, Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, Canada and for Dr. Keith Jarvi, Department of Surgery, Division of Urology, Mount Sinai Hospital, Toronto for providing some of the azoospermic samples used in the study.

Control genotype data from the Illumina OmniExpress platform for 100 men were kindly provided by The Collaborative Study on the Genetics of Alcoholism (COGA); we thank the COGA group and provide a full list of COGA participants in Text S1.

This study makes use of data generated by the DECIPHER Consortium. A full list of centres that contributed to the generation of the data is available from http://decipher.sanger.ac.uk and via email from decipher@sanger.ac.uk.

Author Contributions

Conceived and designed the experiments: AML DTC KIA DFC. Performed the experiments: KIA AML DFC FC JG SF MS AB MJN ET CS AC. Analyzed the data: DFC AML KIA RM NH ET JMD JD AR ABW. Contributed reagents/materials/analysis tools: AML CO XG DAP PNS JS ZH SM IQ JDS DFC DTC. Wrote the paper: DFC. Participated in the interpretation of results: AA MEH. Assisted with writing the manuscript: KIA DTC AML CO JDS PNS.

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Int Urol Nephrol (2012) 44:61–70
DOI 10.1007/s11255-010-9879-4

UROLOGY – REVIEW

Importance of hypogonadism and testosterone replacement therapy in current urologic practice: a review

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Received: 7 July 2010 / Accepted: 10 November 2010 / Published online: 9 December 2010

Abstract Evaluation of potential candidates for testosterone replacement therapy (TRT) includes a complete medical history, physical examination, and hormonal screening. The choice of testosterone assay is important in clinical decision making. TRT should, in theory, approximate natural endogenous production of the hormone. There is no apparent association between TRT and the development of prostate cancer. The administration of exogenous testosterone is not a means of reversing the aging process in men with normal testosterone levels, but it may offer considerable benefit for those with hypogonadism.

The material contained in this article was originally presented at the annual meeting of the American Urological Association, 19 May 2008, Orlando, Florida, USA © American Urological Association Education and Research, Inc.

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Keywords Testosterone · Hypogonadism · Replacement therapy

Introduction and definition

The hormone testosterone (T) is responsible for normal growth and development of male sex organs and maintenance of secondary sex characteristics. It is the primary androgenic hormone, and its production and secretion are the end products of a series of hormonal and enzymatic interactions and feedback regulatory mechanisms. T deficiency results when the testes fail to produce normal levels of T. Hypergonadotropic hypogonadism is called primary testicular failure. T levels are low, and pituitary gonadotropins are elevated. In secondary, or hypogonadotropic hypogonadism, there is inadequate secretion of pituitary gonadotropins and, in addition to low serum T level, luteinizing hormone (LH) and follicle-stimulating hormone levels are low or low normal (Appendix 1). While prepubertal hypogonadism is generally characterized by infantile genitalia and lack of virilization, the development of hypogonadism after puberty frequently results in clinical complaints such as diminished libido, erectile dysfunction (ED), infertility, gynecomastia, impaired masculinization, changes in body composition (muscle mass and fat-to-lean tissue ratio), reductions in body and facial hair, and osteoporosis/osteopenia. In addition to these complaints, mood inventory scores indicate

that hypogonadal men report levels of anger, confusion, depression, and fatigue which are significantly higher than those reported by men with normal T levels.

Age-related declines in serum testosterone

Although wide interindividual variations exist, mean total (TT) and free T levels decline with age, whereas dihydrotestosterone (DHT) and estradiol levels tend to remain relatively constant.

At age 75 years, the mean TT level in the morning is about two-thirds of the mean level at aged 20–30 years, whereas the mean free T and bioactive T (free T plus albumin-bound T) levels are only 40% of the mean levels in younger men. Furthermore, the circadian rhythm of serum T levels is generally lost or attenuated in elderly men [1].

Effects of testosterone deficiency on male sexual function

Sexual interest and activity, and erectile rigidity and duration decline in men as they age. ED is seen with an age-stratified incidence of 1.9% at 40 years and 25% or greater by age 65 [2]. The reported incidence of endocrinopathy as the etiology of ED is 1–35% [3]. Interestingly, the prevalence of abnormally low serum T levels, even among men with ED, has historically been reported to be low (in urology $\sim$6%). The majority of studies show that ED has a clear association with aging, but no consistent correlation of TT with erectile function has been identified. The role T plays in human penile erectile physiology is unclear and so far appears to be different from the effects in animal models. The physiology of erection depends on the integrity of corporal smooth muscle [4]. In a variety of experimental conditions, orchiectomy has been associated with decreased smooth muscle content and increased interstitial collagen in the penis [5]. At the cellular level, the absence of T reduces nitric oxide synthase and nitric oxide production. Recent clinical recommendations have been made regarding assessment of T levels in patients in whom phosphodiesterase type 5 inhibitor therapy fails. Although no direct link has been made to cavernosal muscular atrophy or penile neurological control, it may be that T not only affects sex behavior but also subtly changes a number of physiological parameters directly regulating erectile activity.

Diagnosis of hypogonadism

The diagnosis of hypogonadism requires the art and science of medicine. Subjective symptoms suggestive of androgen deficiency with characteristic signs on physical examination may lead one to suspect hypogonadism, which can then be confirmed by testing and demonstrating low levels of serum T. The hallmark symptom of low T is diminished libido, which, taken in conjunction with signs of testicular atrophy on physical examination, is highly specific for hypogonadism when observed in patients with ED. However, focusing on these men alone may lead one to miss many men who are androgen deficient and who would be well served by T replacement therapy (TRT). Additional sexual symptoms due to low T include ED, difficulty achieving orgasm, reduced intensity of orgasm, diminished ejaculatory volume and reduced sexual sensation in the genital region, particularly the penis. Non-sexual symptoms of hypogonadism include reduced sense of vitality or ‘‘energy,’’ increased fatigue, depressed mood, reduced motivation, and decreased muscle mass or strength. Note that these symptoms are not specific to androgen deficiency, and so a high index of suspicion should lead one to a diagnostic algorithm that would correctly diagnose the cause of these broad symptoms. Signs of androgen deficiency include testicular atrophy, anemia, reduced bone mineral density (osteopenia or osteoporosis), and changes in body composition (increased fat mass and reduced lean body mass). Low T has also been associated with the metabolic syndrome, a set of risk factors for cardiovascular disease [6].

Screening questionnaires on hypogonadism in men are gradually improving. The more commonly used screeners are the Aging Male Symptoms Scale (AMS), Androgen Deficiency in the Aging Males Questionnaire (ADAM), and Massachusetts Male Aging Survey Questionnaire (MMAS), which are intended for the screening or diagnosis of hypogonadism as well as for the evaluation of its therapeutic results. Morley compared these questionnaires in 148 men using bioavailable T as the ‘‘biochemical gold

standard'' for the diagnosis of hypogonadism, and the sensitivity for the ADAM was 97%, for AMS 83% and the MMAS 60%. Specificity was 30% for the ADAM, 59% for MMAS, and 39% for AMS. ADAM and AMS may be useful screening tools for hypogonadism [7]. There has been a lack of consensus as to what blood levels of T should be considered low and, thus, consistent with the diagnosis of hypogonadism. In the absence of a definition of low T, based on the best level of evidence, we must rely on recommendations from expert panels. There have been a number of consensus panels published in the literature, most of which revolve around a level of T of 300 ng/ml as normal. The recently published Endocrine Society Practice Guidelines recommend that TT levels 300 ng/dl be considered diagnostic of hypogonadism and that higher levels be considered normal [8]. However, all attempts to identify a threshold that accurately distinguishes men with hypogonadism have been unsuccessful, and this threshold remains quite arbitrary. Other groups have suggested other thresholds, such as 354 ng/dl by the International Society for the Study of the Aging Male. Given the variability of individual sex hormone–binding globulin (SHBG) levels, a therapeutic T trial may be considered for symptomatic men with low levels of TT or with levels in the low-normal range. This is known as the ''art'' of medicine. Total serum T is composed of three components added together. Roughly half of T is bound to the carrier molecule SHBG, almost all of the remainder is bound to albumin, and *1–2% is unbound or free. A key concept is that T binds so tightly to SHBG that it is functionally unavailable to the cells. In contrast, albumin-bound T-dissociates readily, meaning that this component and the free component are available to cells. The term ''bioavailable testosterone'' refers to a combination of the albumin-bound and free portions [9]. There are a variety of available laboratory tests of T (TT, free T, bioavailable T) that can be ordered as well as the laboratory methods used to determine the actual T level (equilibrium dialysate method for the determination of free T being most accurate). Most laboratories will offer tests for TT and free T. Since SHBG can vary considerably in men, TT levels may be greatly affected. In some cases, relying on TT as an indicator of the adequacy of circulating androgens available for physiological effects will lead to failure to discover hypogonadism. For example, SHBG increases with age, meaning that older men will tend to have normal levels of TT even if they are truly hypogonadal, with low levels of free or bioavailable T. Conversely, obesity is associated with low SHBG, which drives down TT, even when the bioavailable fraction may be normal. The best way to obtain an accurate free T is to use a laboratory offering the equilibrium dialysate method. Alternatively, an acceptable calculation based on TT, SHBG, and serum albumin levels is available. Be aware that laboratory reference values for T vary widely from one institution to another and, thus, are not particularly helpful. In one survey, the reference value used to identify a value as low varied by 350% across 25 laboratories. Therefore, reference values provided by your local laboratory should be considered with this variance in mind. Despite the confusion about what a low T level is, a symptomatic patient, with a low T (or a low normal at my own institution), should be considered for androgen replacement therapy. Therefore, some clinical guidelines and recommendations can be made as a general rule of thumb. Men with TT $<$ 200 ng/dl clearly are hypogonadal, men with TT [400 ng/dl] are unlikely to be hypogonadal and men with TT 200–400 ng/dl should be evaluated based on clinical presentation.

Technical aspects of testosterone assays

Reproducible and cost-efficient testosterone assays that reflect accurate or "true" serum concentrations of T are important in order to correctly diagnose hypogonadism and provide adequate follow-up during therapy. However, with the development of radioimmunoassay (RIA) in the late 1960s, which allows clinicians to easily measure serum T levels, scientific evidence indicates that most testosterone measurements in typical clinical laboratories may be $\geq$30% different from the "true" serum testosterone concentrations measured using gold standard assay methods [10, 11].

The accuracy of measurement is especially important in adult hypogonadal men, where 30% variability may effectively miss a diagnosis of hypogonadism [12]. This is also the case in adolescents where T levels are low and may be below the limit of detection. The need for improved T measurement accuracy is widely accepted among endocrinologists, clinical pathologists, and urologists. The Center for

Disease Control (CDC), in collaboration with industry and major medical associations, has made consensus recommendations geared to improve the quality of T measurements [13, 14].

Poor reliability of T measurements is secondary to both technological limitations of currently available assays, diurnal variation, and the wide range of normal testosterone levels in human samples that assays are required to measure [15, 16]. T levels as low as 10 ng/dl in preadolescents and up to 1,800 ng/dl in young adults are frequently encountered [17].

The most reliable T assays require testosterone to be extracted or displaced from its binding proteins (SHBG and albumin) [18]. This can be accomplished by organic extraction with diethyl ether and precipitation of the remaining proteins. Hence, the binding of T to its carrier protein represents a biological challenge in assays that measure T.

Most currently available assays are based on competitive binding of T in the patient's serum mixed with a set amount of tracer T (radioactive) and fixed amount of antibody against T. (Fig. 1) Because commercially available kits use set amount of tracer and antibody with the goal to measure a wide range of T levels, the ability to accurately measure very low and very high testosterone levels is somewhat poor. For this reason, the age of the patient needs to be provided to the laboratory when T is ordered. There are specialized methods designed for measuring low levels of T in children and women [18]. The detection of low levels of T is achieved by increasing the amount of antibody and decreasing the amount of tracer (Fig. 2).

The RIA has been used for decades, and most of our current normal ranges are based on RIA results. The RIA exhibits a variability of less than 10% in an experienced laboratory with low limit of detection less than 10 pg [10]. However, RIA assays require an experienced staff, produce large amounts of radioactive waist, and are somewhat labor intensive. Thus, radioactive tracers have largely been replaced by enzyme-linked immunoassays (EIA) that detect the amount of bound tracer by detecting a change in color or light (chemiluminescence). Although those assays avoid radioactivity and are easy to automate, they are also based on competitive binding to antibody, suffer from a lack of linearity in a wide spectrum of concentrations, and do not necessarily measure true TT as no testosterone extraction is performed. Both RIA and EIA require specific antibodies with minimal cross-reactivity. Unfortunately, antibodies against steroids are difficult to make, as steroids are poor antigens. Each diagnostic company uses a different antibody, which may in turn have a different activity. This is one reason why standards need to be used in each run with a new batch of kits. Currently available assays lack traceability and national standardization necessary for portability and comparability of results obtained in the same patient [13].

Since it is commonly accepted that only 2% of circulating T, so-called free T (FT), is available to end organs and thus active, it is not surprising that FT should reflect more accurately on the biological effects of T [19, 20].

Unfortunately, no cost-efficient, simple, widely available, reliable, or accurate method of directly measuring FT exists. If one is concerned about low detection limits for total testosterone, the issue becomes even more pronounced for FT when concentrations that have to be measured are 50–100 times lower than TT. Similarly, FT needs to be separated from bound testosterone in order to be measured. This is accomplished by equilibrium dialysis where the patient sample is bathed in dialysis fluid with a known concentration of T. The shift in concentration across membranes is then measured by RIA [21]. More recently, with the use of specialized semipermeable membranes and ultrafast centrifuges, FT can be separated from bound T by centrifugation [22, 23]. Due to equipment complexity and the need for constant rigorous calibration of pores and speed of rotors, these methods are used to validate the indirect methods of FT measurements [24].

Free T is calculated, in most laboratories, based on the concentration of SHBG and albumin and the measured TT concentration. The calculated method correlates with direct concentration of FT, except in certain metabolic situations such as obesity, hyperthyroidism, and low levels of cortisol [25].

Most authorities recommend using TT to screen for hypogoandism and only use FT as a secondary measure to clarify uncertain cases. Direct methods of T assay have gained popularity over the last decade due to both biological and technological factors having such a significant impact on T measurement using immunoassays. The most clinically important method of direct measurement of T, as opposed to indirect immunoassays, is liquid chromatography

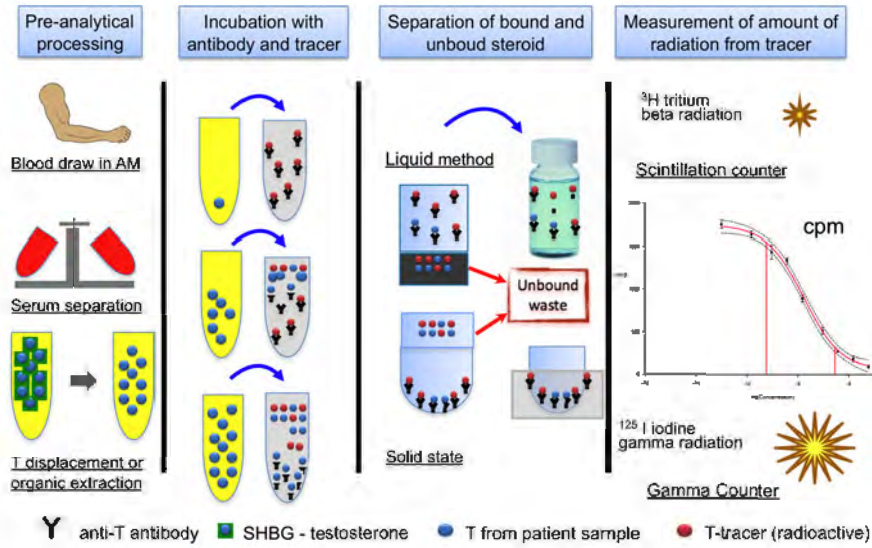

Fig. 1 The principle of a radioimmunoassay is to measure total testosterone (*TT*). The testosterone from serum is mixed with a fixed and known amount of tracer. Testosterone tracer can be marked by 3^{H} tritium or ^{125}I radioactive iodide. Subsequently fixed amounts of antibody raised against T are added, and the mixture is allowed to incubate overnight to allow for equilibrium binding between the patient's T, T tracer and antibody. Depending on the method used, unbound testosterone is absorbed by using activated charcoal and leaving the antibody–ligand in solution. The unbound androgens are removed from the supernatant, and the antibody is linked and remains at the bottom of the reaction tube. The more radioactive tracer displaced by patient's T, the lower the amount of radioactivity measured. Radioactivity in a sample is measured and plotted on a standard curve

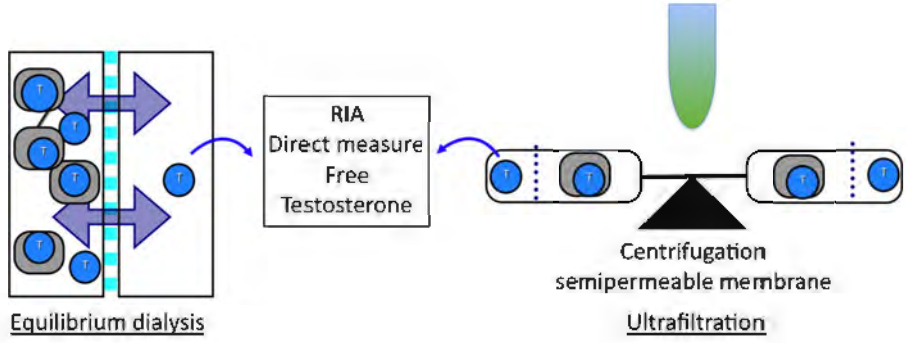

Fig. 2 Free testosterone is measured by equilibrium dialysis (*left*) or ultracentrifugation. Most clinical laboratories calculate FT based on total testosterone and concentrations of SHBG and albumin

tandem mass spectroscopy (LC–MS) [13]. This assay is based on initial separation by liquid chromatography of extracted TT, followed by measurement of the area under the curve to reflect T concentration in the sample [26]. This approach avoids internal problems of specificity and linearity of detection that are observed in immunological assays–even low T concentrations can be detected–as long as an adequate volume of specimen is provided [27]. This specificity is enhanced by a distinct mass spectrometry finger print for each of the known and studied steroids.

LC-MS is the most reliable method of measuring TT in women, hypogonadal men, and children [18, 28].

The normal range of serum total testosterone: epidemiological perspective

The availability of accurate, quick, and reliable methods of T measurement should allow researchers to establish accurate age-appropriate normal ranges of testosterone in a wide population of healthy men.

Currently available norms differ by 200 ng/dL, as some laboratories report low levels of TT as high as 400 ng/dL and others as low as 180 ng/dL. This magnitude of variability is unacceptable in clinical medicine, as it adds the cost of repeating assays, prevents patients from using different laboratories to monitor treatment response, and may lead to diagnostic errors and even unnecessary treatment.

The wide range of norms has its origins in from a lack of reliable and accessible methods to measure TT and a lack of agreement on what constitutes a normal population to base those norms. The majority of studies based their norms on longitudinal population studies that were designed to assess risks for coronary artery disease and not to establish the natural history of sex hormone levels in men. Sex hormones by definition are responsible for normal sexual and reproductive function. To our knowledge, there is no large cross-sectional study that has been used to measure sexual function in healthy men. Boyce used an extended definition of health and noticed that in young men (<30 years of age), 95% CI was much higher than commonly reported norms (582–699 ng/dL) [29]. Sikaris established reproductive hormone norms based on the LC/MS method and semen analysis in 124 men between 21 and 35 years of age; however, he did not evaluate sexual function in this study population. Based on his study, the mean concentration of T was 524 ng/dL (SD: 141 ng/dL), 95% CI: 350–700 ng/dL. Using non-parametric transformation, the norms were calculated from 300 to 864 ng/dL [30]. Unfortunately, this study is rather limited because it focused on only one age group (21–35). Nevertheless, the above studies illustrate that testosterone norms need to be based in populations who are in good general health and exhibit normal reproductive and sexual function.

It behooves clinicians to use the most accurate methods of T measurement and critically evaluate the reported norms. This approach should allow for accurate diagnosis and appropriate clinical decision making.

Treatment of hypogonadism and recognized benefits

Testosterone replacement therapy is indicated for the treatment of T deficiency in aging men with signs and symptoms of hypogonadism. The aims of such treatment are to restore body composition, bone density, libido and mood. One of the major secondary causes of osteoporosis in men is hypogonadism, which is found in up to 20% of men with symptomatic vertebral fractures and 50% of elderly men with hip fractures [31]. A significant increase in trabecular and cortical bone mineral density is documented in the course of TRT, with a slight but significant increase also in paraspinal muscle area. Bone mineral density in long-term regularly treated hypogonadal men is not different from the age-matched reference range for normal men. TRT results in adequate bone mass accumulation and maintenance of normal bone mineral density. By provision of sustained physiological levels of T, they may contribute to an increased androgen effect at the receptor level [32].

However, the ideal dose is difficult to determine because the amounts of T required for androgen-dependent processes are unknown. Evidence suggests that there are different dose–response curves for different androgen-dependent functions. Sexual function (libido) appears to be normalized by relatively low TRT levels, but it is not known whether these levels can maintain bone and muscle homeostasis. Higher levels of T are more likely to have adverse effects on behavior, lipid levels, insulin sensitivity and prostate growth. Available T preparations are listed in "Appendix 2".

Risks of testosterone therapy

All medical therapies are associated with possible adverse effects, and TRT is no exception. The most common concern, particularly among urologists, is the possibility that TRT may also increase the subsequent risk of prostate cancer. The other major issue with TRT is there might be an increased risk of cardiac disease. Neither of these concerns appears to have a scientific basis. Multiple studies have shown that the effect of TRT on the lipid profile is neutral. Transdermal therapy, in particular, has little to no effect on serum lipids in most studies and when present changes have generally demonstrated a balanced effect, for example a reduction in HDL (the ''good'' cholesterol) together with a decrease in LDL (the ''bad'' cholesterol). For many years, it was believed that TRT may increase the risk of heart

disease, since men have a higher incidence of cardiovascular events than women. However, as newer evidence accumulates, we find that the opposite may be true. Several studies suggest that higher levels of T may actually confer a favorable effect on the risk of cardiovascular disease, and therapeutic reduction in androgens may lead to significant risks of cardiovascular incidents and even death. For example, in the Rotterdam study [33], men with T levels in the highest third had a risk of atherosclerosis of the abdominal aorta that was only 20% as high as men with T in the lowest third of the population.

In a small study, English et al. [34] reported that 22 men with chronic stable angina treated with transdermal TRT had greater angina-free exercise tolerance compared to 24 placebo-treated controls. Furthermore, direct injection of physiological levels of T into the coronary arteries led to an increase in mean coronary artery diameter and blood flow compared to baseline. Studies of TRT have not demonstrated an increased incidence of cardiovascular disease or events such as myocardial infarction, stroke, or angina episodes. A recent meta-analysis of 30 trials comprising 1,642 men revealed that TRT is not associated with detrimental cardiovascular events [35].

Low levels of T in men have been shown to be associated with type 2 diabetes, visceral adiposity, dyslipidemia, and metabolic syndrome. TRT reduces insulin resistance and improves glycemic control in hypogonadal men with type 2 diabetes. Improvements in glycemic control, insulin resistance, cholesterol and visceral adiposity together represent an overall reduction in cardiovascular risk [36].

Although the available data appear reassuring, definitive assessment of the long-term consequences of TRT on cardiovascular health will require prospective, large-scale, placebo-controlled studies. Interestingly, there is emerging evidence that low T levels, such as those obtained with androgen suppression therapy (AST) for prostate cancer, may actually increase the risk of cardiovascular morbidity and mortality. D'Amico et al. [37] investigated the influence of androgen suppression therapy for prostate cancer on the frequency and timing of fatal myocardial infarctions (MI) and found an earlier onset of fatal MIs in men 65 years old or older on AST for 6 months or more. Laughlin et al. [35] examined the association of endogenous T levels on 794 older community-dwelling men followed for an average of 11.8 years. They reported that T deficiency in older men was associated with an increased risk of death during a 20-year period and that the increase in risk was independent of multiple cardiovascular risk factors or preexisting health conditions. The greatest concern regarding TRT is the fear that higher T levels will increase the risk of prostate cancer. Specifically, the concern is that small, undetected, inactive or incidental tumors will progress into clinically worrisome disease. This concern stems from the well-known fact that prostate cancer is androgen dependent. However, after age 65 years, there remains no compelling evidence that this risk is real. Multiple studies have failed to show any association between higher T and subsequent risk of prostate cancer.

A study on a large prospective cohort of 10,049 men contributes to the gathering evidence that the long-standing ''androgen hypothesis'' of increasing risk with increasing androgen levels can be rejected, suggesting instead that high levels within the reference range of androgens, estrogens, and adrenal androgens decrease aggressive prostate cancer risk. Indeed, high-grade prostate cancer has been associated with low plasma level of T. Furthermore, pretreatment TT was an independent predictor of extraprostatic disease in patient with likelihood of non-organ confined disease, and low serum T levels are associated with positive surgical margins in radical retropubic prostatectomy [38].

Testosterone replacement therapy in men at high risk for prostate cancer due to preexisting prostatic intraepithelial neoplasia did not demonstrate any increased risk of prostate cancer.

Marks et al. [39] provide a good explanation of why TRT does not appear to increase prostate cancer risk. In a placebo-controlled trial, men received intramuscular T every 2 weeks for 6 months. Tissue levels of T and DHT were obtained by biopsy before TRT and at the end of the 6-month trial. Although serum levels of T and DHT increased substantially, there was no change in these androgens within the prostate itself. Indeed, there is now some concern that men with low T may be at increased risk of prostate cancer. Prostate biopsy in hypogonadal men with prostate-specific antigen (PSA) <4.0 ng/ml revealed cancer in 15%, and men with more severe reductions in T had a risk of cancer that was double the risk for

those with milder reductions in T. Low T has been associated with worse prognosis, higher disease stage at presentation and higher-grade cancers. Another less significant risk of TRT is preexisting benign prostatic hyperplasia. TRT causes a mild increase in prostate volume and PSA of ~15% but does not appear to worsen voiding symptom scores, uroflow values, or post-void residual volumes in this group. Another common adverse effect of TRT is erythrocytosis, representing an elevation in the hematocrit or hemoglobin. This is more likely to occur in men living at high altitude (Rocky Mountain States) or those who are on parenteral therapy. On average, TRT causes an increase in the hematocrit of ~3%. In a clinical trial of intramuscular testosterone, at least 1 episode of increased hematocrit occurred in 44% of men compared with 15% in men receiving testosterone via a transdermal patch. Proper monitoring and dose adjustment are necessary in men at risk of erythrocytosis with increased hemoglobin/hematocrit levels. Gynecomastia is an uncommon event associated with TRT, occurring via conversion of testosterone to estradiol, and acne may occur in a small percentage of men due to increased oiliness of the skin from higher testosterone levels. TRT has been associated with new onset or exacerbation of sleep apnea. The mechanism for this relationship remains unclear, but men beginning therapy should be questioned for a preexisting diagnosis of sleep apnea. Oral forms of testosterone have been associated with liver toxicity and are strongly discouraged. Specifically, intramuscular injections, patches, and gels appear safe with regard to the liver. There is no need to monitor liver function tests in men receiving these more standard modes of therapy. Testicular atrophy and infertility are important effects of TRT. Exogenous testosterone down-regulates release of LH and follicle-stimulating hormone. While serum levels may normalize, the interstitial environment within the testicle exhibits dramatically lower relative levels because the Leydig cells that are intimately associated with the spermatogenic epithelium are no longer producing T, resulting in dramatic decreases in sperm production. Most men receiving TRT will be azoospermic or have severely depressed sperm concentrations, which usually return to baseline after cessation of TRT. Reduction in spermatogenesis may also result in reduced testicular size, which may be more noticeable and of greater importance to younger men. It is necessary to ask any man who is a candidate for TRT whether he desires children in the near future and, if so, he should not receive TRT. Some of these men may be successfully treated with agents that increase endogenous production of T, such as clomiphene citrate and gonadotropins.

About the clinical management of TRT, there are useful guidelines [40].

Clinical summary

T is the primary androgenic hormone responsible for normal growth and development of male sex organs and maintenance of secondary sex characteristics. Evaluation of potential candidates for TRT includes a complete medical history, physical examination, and hormonal screening. Total serum T should ideally be measured in the morning. When the serum T level is low and LH is elevated, TRT is warranted as this suggests primary (hypergonadotropic) hypogonadism. When serum levels are low, LH is normal or low and prolactin levels are elevated, an imaging study of the pituitary region is warranted, and endocrinology consultation may be needed.

Testosterone replacement therapy should, in theory, approximate natural endogenous production of the hormone. The clinical rationale for the treatment of T deficiency may include stabilizing or increasing bone density, enhancing body composition by increasing muscle strength and reducing adipose tissue, improving energy and mood, and maintaining or restoring secondary sexual characteristics, libido, and erectile function. The physician prescribing TRT must evaluate for any changes in the clinical symptoms and signs of T deficiency and must monitor the patient regularly by performing digital rectal examination and checking serum T levels, PSA, and hematocrit at baseline and at prescribed intervals (at 3–6 months and then annually) during treatment. Although TRT is contraindicated in men with carcinoma of the breast or known or suspected carcinoma of the prostate, in general, therapy appears to be safe for the majority of hypogonadal men. There is no apparent association between TRT and the development of prostate cancer. The administration of exogenous T is not a means of reversing the aging process in men with normal T levels, but it may offer considerable benefit for those with hypogonadism.

Appendix 1

Primary hypogonadism

(↓ T and ↑ LH)

- Klinefelter's syndrome
- Mumps orchitis
- Autoimmune orchitis
- Trauma
- Testicular irradiation or surgery

Secondary hypogonadism

(↓ T and ↓ ↔LH)

- Acquired idiopathic
- Pituitary tumors
- Uremia
- Systemic illness
- Cranial irradiation
- Hyperprolactinemia
- Hemochromatosis
- Cushing's syndrome
- Cirrhosis
- Morbid obesity
- Metabolic syndrome
- Diabetes mellitus

Appendix 2

Testosterone preparations

- Oral agents
- Pellet implants
- Scrotal patches
- Intramuscular preparations
  - Short acting
  - Long acting
- Transdermal patches
- Transdermal gels
- Buccal tablets

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Journal of Andrology, Vol. 33, No. 5, September/October 2012

Methylation-Specific PCR Allows for Fast Diagnosis of X Chromosome Disomy and Reveals Skewed Inactivation of the X Chromosome in Men With Klinefelter Syndrome

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ABSTRACT: Klinefelter syndrome (KS) remains the most common, yet often undiagnosed, chromosomal aberration in men. Early diagnosis and treatment can improve the health of patients with KS. The aim of this study was to evaluate the inactivation pattern of supernumerary X chromosomes. The secondary aim was to design a reliable and cost-effective molecular test for detection of X chromosome disomy. Methylation-specific polymerase chain reaction (M-PCR), with primers for familial mental retardation (FMR1) and X chromosome inactive-specific transcript (XIST) genes, was used to detect the presence of X chromosome disomy in men. Seventeen fertile males, 12 females, and 35 males with KS (28 with 47,XXY karyotype, and 7 with 47,XXY/46,XY mosaics) were included in the study. Results of the karyotype were compared with the results of semiquantitative M-PCR. Inactivation of X chromosomes was measured by XIST/FMR-1 methylation ratio. Differences in the methylation patterns of FMR1 and XIST genes between 46,XY men and men with X chromosome disomy allowed for rapid detection of the presence of an additional X chromosome, achieving 100% sensitivity and specificity using M-PCR. The methylated:unmethylated FMR1 amplicon ratio allowed the detection of 1 additional X chromosome per 100 normal XY cells (1% of XX/XY mosaicism). In our series, 50% of 47,XXY men showed skewed inactivation of the X chromosome. Men with KS can have incomplete inactivation of supernumerary X chromosomes. M-PCR is a sensitive, specific, fast, and relatively inexpensive test for the diagnosis of X chromosome disomy.

Key words: Polymerase chain reaction, prenatal genetic diagnosis.

J Androl 2012;33:955–962

Klinefelter syndrome (KS) is the most common numerical chromosomal aberration in men, with an estimated frequency of 1:500 to 1:1000 live deliveries (Nielsen and Wohlert, 1990). It is characterized by X chromosome polysomy, with X disomy being the most common variant (47,XXY). Ninety percent of men with KS have nonmosaic X chromosome polysomy (Lanfranco et al, 2004). Nevertheless, there is considerable phenotypic variation among men with KS, with the classic findings of eunuchoid body proportions, sparse facial and pubic hair, small and firm testicles, and severe intellectual deficits being less common than previously described. Possible explanations for this phenotypic variation include differences in hormonal profiles, differences in genetic background, and abnormal inactivation of supernumerary X chromosomes.

It has been noted that men with more than 2 X chromosomes (48,XXXY; 49,XXXXY) are more severely affected than men with the classic 47,XXY karyotype (Paduch et al, 2008). The presence of 2 active X chromosomes (X ch) in animals and hybridoma models is lethal (Heard and Disteche, 2006), and inactivation of 1 X ch is critical to achieve normal development (Nguyen and Disteche, 2006). In normal females, 1 X ch randomly undergoes inactivation in embryonic tissues; it is only logical that a similar mechanism also occurs in men with X ch polysomy. The X ch bears more than 1100 genes critical for normal function of the brain and testes (Ross et al, 2005). Overexpression of X ch–linked genes could be responsible for cognitive impairment and spermatogenic failure seen in 47,XXY men (Vawter et al, 2007).

Inactivation of the supernumerary X ch is initiated within the X chromosome inactivation center (XIC), by activation of the X chromosome inactive-specific transcript (XIST) promoter (Hong et al, 2000). Transcription of XIST RNA allows for multifocal painting of the X ch and subsequent recruitment of inactivation

Supported by The Frederick J. and Theresa Dow Wallace Fund of the New York Community Trust and the Robert Dow Foundation.

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Received for publication November 30, 2011; accepted for publication March 14, 2012.

DOI: 10.2164/jandrol.111.016030

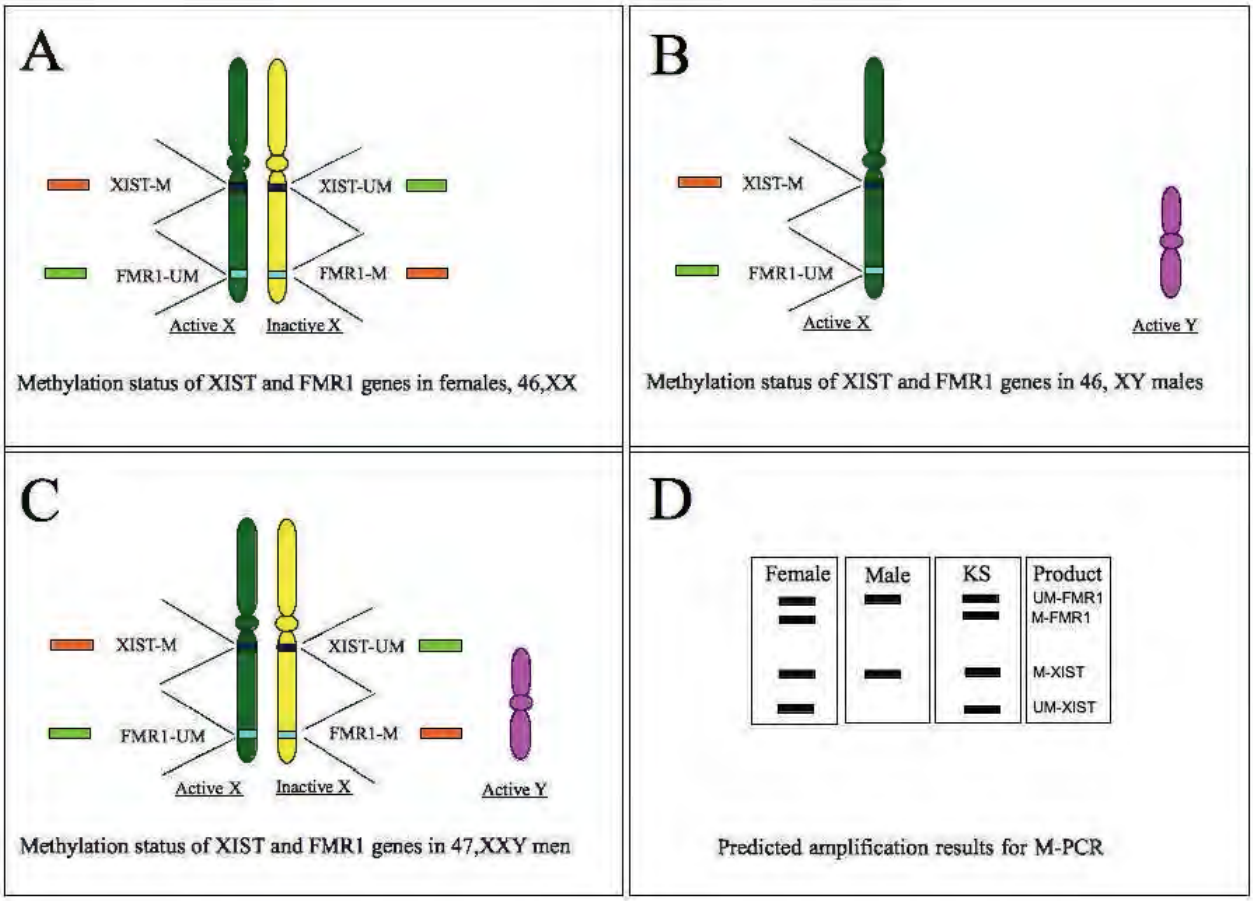

Figure 1. Differences in methylation (M) of familial mental retardation (FMR1) and X chromosome inactive-specific transcript (XIST) genes between females **(A)**, normal males **(B)**, and patients with Klinefelter syndrome (KS) **(C)** result in different patterns of amplification of genes of interest **(D)**. In 46,XY men, their only X chromosome has to remain active. Therefore, on electrophoresis of polymerase chain reaction (PCR) products, only 2 products will be visible: 1 from unmethylated and transcriptionally active (FMR1-UM) (green), and one from XIST-M (orange), whereas females and patients with KS will have 4 products after methylation-specific PCR (M-PCR).

proteins with H3 and H4 deacetylation and methylation, linking the expression of XIST to chromatin remodeling and gene silencing (Matarazzo et al, 2008). Because of this multistep mechanism of X ch inactivation and the escape of some genes from X ch inactivation, it is possible that abnormal or skewed inactivation of supernumerary X chromosomes in men with X ch polysomy leads to the cognitive and reproductive problems seen in this group.

Application of methylation-specific polymerase chain reaction (M-PCR) in the detection of X ch disomy is based on the differences in methylation of the familial mental retardation (FMR1) gene between females and males and the evolutionary principle of dose compensation, which equalizes phenotypic expression of characteristics determined by X chromosome genes. To avoid excess FMR1 protein (mental retardation), only 1 copy of the FMR1 promoter is unmethylated and transcriptionally active (FMR1-UM) at any time; in females, the second copy is inactivated through methylation of CpG islands (FMR1-M). Although the mechanism is poorly understood, it is believed that genes located in XIC are responsible for the detection of an additional X chromosome and for XIST transcription. In turn, XIST binds to its specific site on supernumerary X ch and turns off gene transcription (Escamilla-Del-Arenal et al, 2011; Heard and Turner, 2011; Werler et al, 2011). In normal males, XIST is methylated and, therefore, transcriptionally inactive; in females, 1 copy of XIST is methylated (inactive) and the other is not (active). The XIST gene, therefore, has the opposite pattern of methylation to that of FMR1. Females and KS males are expected to have similar inactivation patterns of XIST and FMR1 genes and, thus, the same amplification pattern on gel electrophoresis after M-PCR (Figure 1).

Screening for KS in target populations is limited by the cost of established tests for KS diagnosis, such as karyotype analysis and quantitative real-time polymerase chain reaction. Recently, Barr body cytology has been proposed as a cheaper test for KS screening, but

Table 1. *Characteristics of the primers used for methylation-specific polymerase chain reaction*

Primer	Sequence of Primer	Concentration, μM	Position	GenBank
FMR1-R	5′-ATTTAATTTCCCACRCCACTAAATACAC-3′	0.4	13395–13422	L29074 L38501
FMR1-UM-L	5′-GTGTTTGATTGAGGTTGAATTTTTG-3′	0.2	13712–13688	L29074 L38501
FMR1-M-L	5′-GTTGCGGGTGTAAATATTGAAATTACG-3′	0.2	13683–13657	L29074 L38501
XIST-M-L	5′-AATTAAAGTAGGTATTCGCGGTTTCG-3′	0.32	19049–19024	U80460
XIST-M-R	5′-TTTTTCCTTAACCCATCGAAATATCG-3′	0.32	18834–18809	U80460
XIST-UM-L	5′-AAAAGTGGTTGTTATTTTAGATTTGTT-3′	0.32	19238–19260	U80460
XIST-UM-R	5′-CTACCTCCCAATACAACAATCACAC-3′	0.32	19435–19411	U80460

Abbreviations: FMR1-R, familial mental retardation common primer; FMR1-UM-L, unmethylated left primer; FMR1-M-L, methylated left primer; XIST-M-L, X chromosome inactive-specific transcript methylated left primer; XIST-M-R, methylated right primer; XIST-UM-L, unmethylated left primer; XIST-UM-R, unmethylated right primer.

although the test has 95% specificity, its sensitivity is limited to 82% (Kamischke et al, 2003).

Pena first proposed use of FMR1 gene analysis in the diagnosis of KS in a letter to the *Journal of Andrology* in 2003 (Pena and Sturzeneker, 2003). However, to date, there has been no previous publication evaluating this technique in the setting of KS. The objective of this study was, therefore, 2-fold. Using differences in the methylation pattern of 2 genes located on X chromosome, FMR1 and XIST, the primary aim was to evaluate the inactivation pattern of supernumerary X chromosomes in men with KS. The secondary aim was to develop a cost-effective, rapid, and reliable method of KS diagnosis based on the molecular mechanism of X ch inactivation.

Materials and Methods

This retrospective study was based on an institutional review board-approved deoxyribonucleic acid (DNA) repository. Electronic medical records of patients who had their DNA stored in the repository were reviewed, and patients with a known karyotype were identified. Seven 47,XXY/46,XY males and twenty-eight 47,XXY males were included in this study. Karyotype analysis and assays of serum testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estradiol levels were performed by using a commercial clinical laboratory (ARUP Labs, Salt Lake City, Utah). Testicular volume was measured by a single attending physician using a Prader orchidometer. A DNA sample from all subjects in this project was extracted using a Promega Wizard Genomic DNA purification kit (Promega, Madison, Wisconsin) according to the manufacturer's guidelines and stored at −20°C. Cost analysis was performed using the purchase price for each kit and chemical obtained.

Y Chromosome Detection

After DNA extraction and purification, presence of the Y chromosome was confirmed by PCR with primers specific for short (SY14; UniSTS: 42547) and long arms (SY88; UniSTS: 80503) of the Y chromosome. Genomic template DNA (150 ng) was added to the PCR mixture containing 0.2 μM SY14 primer, 0.32 μM SY88 primer, 12.5 μL of Qiagen Multiplex PCR master mix (Qiagen, Valencia, California), and water for a total of 25 μL of reaction mixture. Fragments were amplified in a multiplex PCR reaction using GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, California). The presence of PCR product was verified by electrophoresis of the PCR product on 4% agarose gel stained with ethidium bromide.

DNA Deamination

DNA was deaminated using a modification of the methods described by Herman et al (1996). Human DNA (0.5 μg) was suspended in 50 μL of double-distilled water and denatured at 37°C for 17 minutes after adding 5.5 μL of 2 M NaOH. Freshly prepared 10 mM hydroquinone and 3 M bisulfite (Sigma-Aldrich, St Louis, Missouri) was added to denatured DNA, and the mixture was placed in a water bath (55°C) for 1.5 hours under PCR-grade oil (Perkin Elmer, Waltham, Massachusetts). The time of denaturation was experimentally derived to allow for a conversion rate of 99%. Deamination was terminated by adding 150 μL of 96% ethanol/3 M NaOH mixture during DNA purification with Qiagen QIAquick PCR Purification Kit (Qiagen, Valencia, California). DNA was eluted with 100 μL of Tris-EDTA buffer and stored at −20°C.

M-PCR

Two sets of primers (methylated and unmethylated) for each DNA template sequence were used for M-PCR (Table 1; Figure 2). Four microliters of template DNA (100 200 ng) was mixed with 2 μL of primer mix and 19 μL of PCR master mix. Master mix was prepared with 2.5 μL of 10× Roche reaction buffer with magnesium (Roche, Basel, Switzerland), 0.2 μL of Stratagene 200 mM dNTP mix (Agilent, Santa Clara, California), 0.25 μL of FastStart High Fidelity Polymerase (Roche), and 16.05 μL of water per each 25-μL PCR reaction. Polymerase chain reaction amplification was performed with GeneAmp PCR system 9700 thermal cycler: 95°C for 3 minutes × 1, (95°C for 30 seconds/61°C for 40 seconds/72°C for 50 seconds) × 45 cycles, 72°C for 7 minutes × 1, and 4°C for

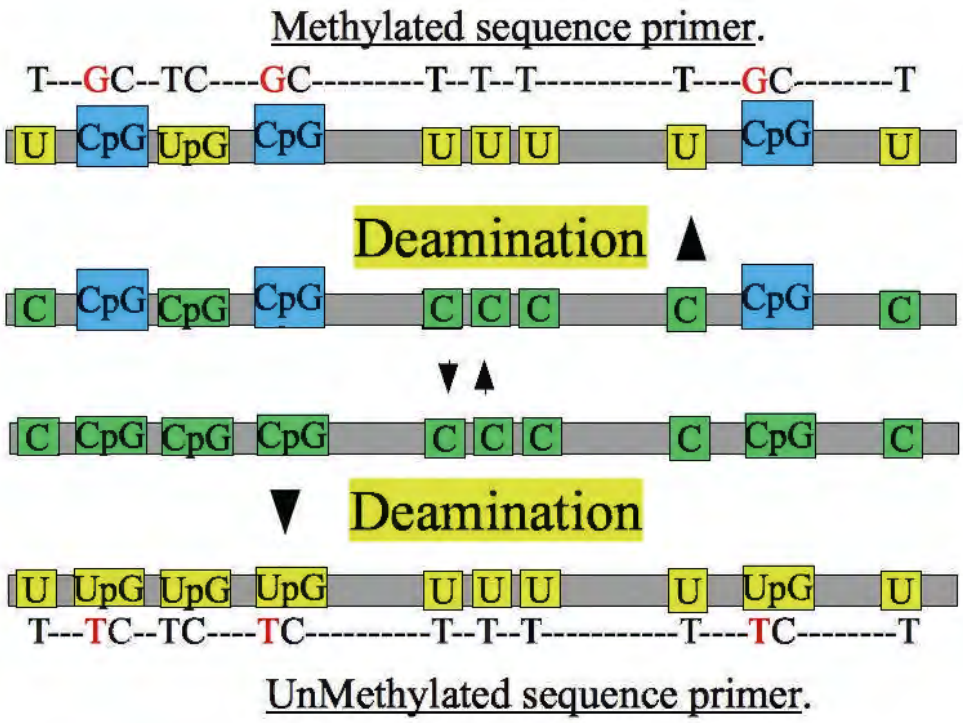

Figure 2. Design of primers for methylation-specific polymerase chain reaction. During deamination, unmethylated cytosines (yellow box) will be converted to uracil (U). Methylated cytosines (blue box) will remain unchanged. Highlighted in red are differences in each primer needed after deamination.

20 minutes. The PCR product was mixed with Blue Juice gel loading buffer (Invitrogen, Carlsbad, California) and loaded on 4% agarose gel stained with ethidium bromide. DNA molecular weight marker VIII (Roche) was used as a size standard.

Sensitivity and Specificity of M-PCR in Detection of X Chromosome Disomy

After optimizing the M-PCR and primer concentrations on 2 male and 2 female controls, M-PCR was performed on 17 fertile male controls, 12 female controls, and 35 males with KS, 28 of whom were 47,XXY and 7 were 47,XXY/46,XY mosaics, verified by karyotype. The primers and PCR settings used are described earlier. The specificity of primers was evaluated with native template DNA (not deaminated) using the same M-PCR setting as for deaminated DNA. All experiments were repeated 3 times.

To determine the lowest percentage of XX/X mosaicism detectable by M-PCR, the DNA from fertile male and female was mixed to achieve XX/X ratios of 50%, 25%, 12.5%, 8%, 5%, 3%, 2%, 1%, and 0.5%. Subsequently prepared samples were deaminated, and M-PCR was performed as described earlier. Each experiment was repeated 3 times to ensure reproducibility. Net optical densities of FMR1-UM and FMR1-M bands were measured using Kodak 1D Image Analysis Software v. 2.02 for Windows (Kodak-Eastman Inc, Rochester, New York).

Pattern of X Chromosome Inactivation

To test the hypothesis that men with Klinefelter syndrome have skewed inactivation of the X chromosome, the FMR1-UM/methylated XIST (XIST-M) ratio was measured in female controls and men with Klinefelter syndrome. Theoretically, the ratio of the unmethylated gene of interest and methylated XIST gene in females should be 1:1 to allow for transcription of material from active X ch. If men with KS have skewed inactivation of the X chromosome, or if the inactivation is promiscuous, then a different ratio of the unmethylated gene of interest to methylated XIST would be expected (Figure 1).

Statistics

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, La Jolla, California). One-way analysis of variance was used to compare the difference in means between the FMR1-UM/XIST-M inactivation ratios in men with KS and female controls. Because the inactivation ratio among men with KS had a bimodal distribution, 3 patient groups (2 groups of men with KS and 1 group of female controls) were used for the final analysis. Tukey's multiple comparison test was used to test for differences in means between groups. A $P \leq .05$ was considered statistically significant.

Results

On average, the 47,XXY and 47,XXY/46,XY men in the study population had low serum testosterone (252.22 ± 115.8 ng/dL) with elevated serum FSH (32.96 ± 12.43

Table 2. *Endocrinological characteristics of men with Klinefelter syndrome (n = 35)*

Serum Hormone	Average Level ± SD	Reference Range
Total testosterone, ng/dL	252.22 ± 115.82	270–1730
FSH, IU/L	32.96 ± 12.43	0.4–8.0
LH, IU/L	18.84 ± 9.01	2.0–12.0
Estradiol, ng/L	23.85 ± 11.16	<60
T/E ratio	11.9 ± 6.1	>20

Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone; T/E, testosterone/estradiol.

IU/L) and LH (18.84 ± 9.01 IU/L) concentrations (Table 2). The average left and right testicular volume was also low, at 3.68 ± 3.11 mL and 4.12 ± 3.3 mL, respectively. The clinical and laboratory profile for these men was typical for KS. The presence of a Y chromosome was positively verified for all 35 men with KS by multiplex PCR using STS markers for both the short and long arm of the Y chromosome.

All 46,XY men had 2 amplicon bands on gel electrophoresis: 1 unmethylated FMR1 promoter amplicon (FMR1-UM) and 1 band corresponding to the methylated XIST gene (XIST-M). All females and 47,XXY males had 4 bands on the gel, reflecting the presence of the following amplicons—methylated and unmethylated FMR1 promoter (FMR1-UM, FMR1-M) and methylated and unmethylated XIST gene (XIST-M, XIST-UM)—thus verifying the hypothesis that M-PCR can detect the presence of X chromosome polysomy (Figure 3; Table 3). The 47,XXY/46,XY males all tested positive for X ch disomy using the M-PCR assay (Table 3). To exclude nonspecific amplification of methylation-specific primers, parallel multiplex PCR with native and deaminated DNA was performed. Although the bands were always visualized using the deaminated DNA, there were no bands using native DNA from the same patients. Analysis of the inactivation pattern among females and men with KS verified

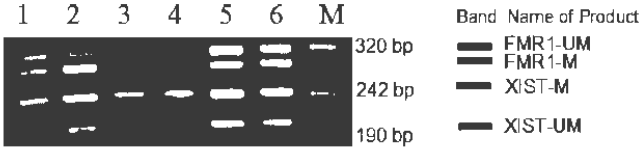

Figure 3. Methylation-specific polymerase chain reaction allows for easy diagnosis of Klinefelter syndrome (KS). Females and patients with KS have 4 bands representing different patterns of inactivation of each of 2 X chromosomes. Normal men have only 2 bands because they have a single X chromosome. 1,2 indicates 2 normal females (46,XX); 3,4, 2 normal males (46,XY); 5,6, two 47,XXY males with KS; M, molecular weight marker; FMR1, familial mental retardation; XIST, X chromosome inactive-specific transcript; M (in Band Name of Product), methylated primer; UM, unmethylated primer.

Table 3. *Results of methylation-specific polymerase chain reaction (M-PCR)*

	46,XY	47,XXY	47,XXY/46,XY	46,XX
Test negative	17	0	0	0
Test positive[a]	0	28	7	12
Total (n)	17	28	7	12

[a] Test is considered positive for Klinefelter syndrome if there are 4 bands on the gel using familial mental retardation (FMR1)/X chromosome inactive-specific transcript multiplex or 2 bands using FMR1 duplex M-PCR only.

that the additional X ch in men undergoes inactivation (based on the methylation pattern of FMR1 and XIST genes).

When karyotype results were compared with those of M-PCR, 100% specificity and 100% sensitivity were achieved in detecting X chromosome disomy in males with nonmosaic KS. M-PCR was able to detect 1% or more of 47,XXY/46,XY mosaicism (Figure 4).

The analysis of X ch inactivation patterns in females and KS males revealed skewed inactivation of the X ch in men with KS. In fertile females, the inactivation ratio (FMR-UM/XIST-M) was 1.0, as expected, whereas in men with the 47,XXY karyotype, inactivation was skewed toward inadequate inactivation of genetic material on the supernumerary X chromosome. The inactivation pattern in men with KS was bimodal, with a ratio of 1.8 as a cutoff point between groups. Fifty percent of men with KS had highly skewed inactivation with a ratio greater than 1.9. There was no statistically significant difference in serum hormone profiles between men with normal and highly skewed X ch inactivation (Figure 5). There was a statistically significant difference in terms of the FMR1/XIST ratio between females and KS males, with an inactivation ratio greater than 1.8, but not between females and KS males with an inactivation ration less than 1.8 (Figure 5).

Material costs to detect the presence of additional X chromosomes in males using methylation-specific PCR were estimated at $5.49 per patient blood sam-

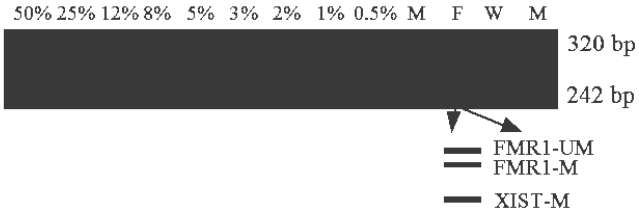

Figure 4. Sensitivity of methylation-specific polymerase chain reaction in detecting XX/XY mosaic. Upper line numbers correspond to the degree of XX/XY mosaicism. F indicates female control; W, negative control (water); M, molecular weight marker; FMR1, familial mental retardation; XIST, X chromosome inactive-specific transcript; M (below arrows), methylated primer; UM, unmethylated primer.

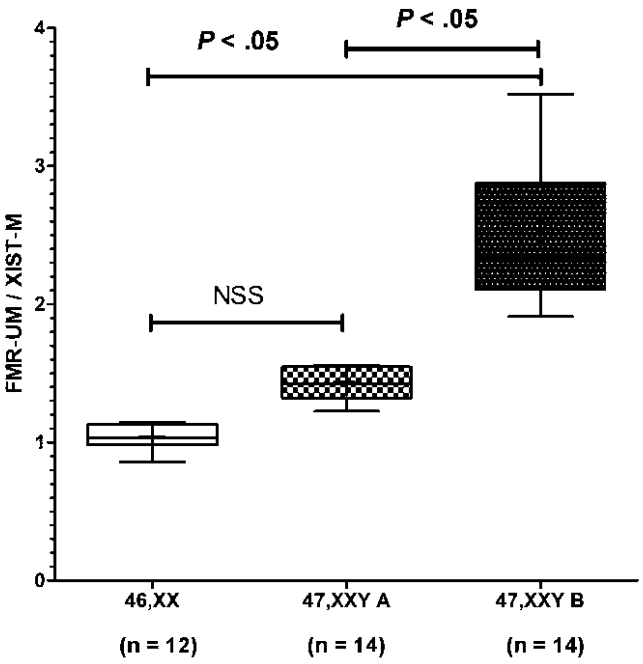

Figure 5. Results of semiquantitative measure of skewed inactivation of X chromosomes using familial mental retardation (FMR1)/methylated X chromosome inactive-specific transcript (XIST-M) ratio. In fertile females, the ratio of inactivation was 1, as expected; in men with 47,XXY karyotype inactivation is skewed toward inadequate inactivation of genetic material on the supernumerary X chromosome. The inactivation pattern in men with Klinefelter syndrome (KS) was bimodal, with a ratio of 1.8 as a cutoff point between groups (group A, ratio $<$ 1.8; group B, ratio $>$ 1.8). Fifty percent of men with KS showed highly skewed inactivation, with ratio $>$ 1.9. There were statistically significant differences in the FMR1/XIST ratio between females and males with KS in-group B. NSS indicates not statistically significant; UM, unmethylated primer.

ple. The turnaround time for M-PCR was less than 48 hours from the time of sample receipt to reporting of results.

Discussion

Although KS is the most common sex chromosomal abnormality in men, most patients remain undiagnosed during their lifetime (Bojesen et al, 2003). The results of this study demonstrate that M-PCR can be used to assess the inactivation pattern of supernumerary X chromosomes in men with KS. The high sensitivity and specificity of M-PCR allow for detection of as little as 1% XX/XY mosaicism. M-PCR is fast and cost-effective, which makes it a valuable tool for the early diagnosis of KS. Our study also showed that X ch inactivation in men with KS is skewed, compared with normal women. This escape from inactivation might contribute to the phenotypic variation seen in affected individuals (Iitsuka et al, 2001).

KS is characterized by low serum testosterone, elevated serum LH and FSH, and infertility from primary testicular failure. Many patients also demonstrate some cognitive impairment and have a higher risk of developing medical comorbidities, such as autoimmune diseases, diabetes mellitus, osteoporosis, tumors, and increased mortality. This clinical phenotype is not typically present in individuals with hypogonadism due to Kallman syndrome, suggesting that abnormal function of X-linked genes, rather than low serum testosterone per se, is responsible for the phenotypic presentation of KS.

The use of M-PCR has been previously described in the diagnosis of fragile X syndrome (Coffee et al, 2009) and KS (Werler et al, 2011). In the present study, differences in the methylation patterns of FMR1 and XIST genes between 46,XY men and men with mosaic or nonmosaic X chromosome disomy allowed for fast detection of the presence of an additional X chromosome, achieving 100% sensitivity and specificity using M-PCR. These results are similar to findings of Coffee et al (2009), who also reported M-PCR as having 100% sensitivity and specificity for detecting methylation of the FMR1 gene. High sensitivity and reliability are clear advantages of the use of M-PCR as a screening tool.

Conventional cytogenetics, or karyotype analysis, is the current gold standard for the diagnosis of KS. One of the shortcomings of this approach is its low sensitivity for the detection of mosaicism (Okada et al, 2001). Our results show that M-PCR is able to detect very low levels of mosaicism. M-PCR could therefore be useful in verification of mosaicism in cases where mosaicism is clinically suspected but karyotype results are negative.

Established tests for the diagnosis of KS, such as karyotype analysis and real-time quantitative PCR, are expensive. Barr body cytology, although cheaper, is limited by sensitivity and specificity of 95% and 82%, respectively (Kamischke et al, 2003). Fluorescence in situ hybridization (FISH) has similar specificity and sensitivity to karyotype analysis but requires expensive probes, an experienced technologist, and imaging software. The average turnaround time for M-PCR in this study from the receipt of samples to availability of results was less than 48 hours, at a cost of less than $6 per sample. Molecular techniques have long been used in the preimplantation genetic diagnosis of various heritable diseases (Sermon et al, 2004). Advantages of PCR-based technology include familiarity with the technology, availability of equipment in virtually every research and clinical laboratory, low volume of blood needed for the DNA extraction, and low cost. Recent literature on prenatal screening for common aneuploidies (13, 18, 21, and sex chromosomes) confirms that molecular techniques are not only as sensitive as

karyotype and FISH but also more economical and efficient (Donaghue et al, 2003; Mann et al, 2004).

Although the benefit of systematic screening for KS has been debated in the literature (Herlihy et al, 2011a), growing evidence suggests that early diagnosis and therapeutic interventions in children with KS could have a beneficial effect on their physical, academic, and social development, as well as their overall health (Simpson et al, 2003; Herlihy et al, 2011b; Samango-Sprouse et al, 2011). Unfortunately, only 10% of men affected by KS are diagnosed during preadolescence and adolescence, the time when treatment can be the most effective (Bojesen et al, 2003). Availability of a rapid and a cost-effective postnatal screening test for KS can be expected to improve significantly the diagnosis and follow-up of individuals with KS.

The results of this study confirm that the supernumerary X ch undergoes inactivation in men with KS. Nevertheless, the presence of supernumerary X chromosomes results in KS, indicating that the process of X ch inactivation is skewed, or that there is selective allelic drop-out from inactivation of certain X ch genes. The regulation of X ch inactivation in men with KS is certainly less stringent than in female controls because the ratio of FMR1-UM/XIST-M was statistically and clinically significantly different between the 2 groups (1.8 vs 1.0, respectively). A high ratio of FMR1-UM/XIST-M indicates that in approximately 50% of 47, XXY men, genetic material may escape methylation and, thus, inactivation. Some degree of skewed X ch inactivation is known to occur in normal females. Genes such as *ZFK*, which code for proteins involved in sperm and oocyte development and have alleles on both X and Y chromosomes, are normally "active" on both the active and inactive X ch in females. Heard and Disteche (2006) have estimated that 15% of X ch genes escape inactivation in normal females, and more often in cancer. Thus, it is possible that in men with KS, some genes on additional X chromosomes escape epigenetic regulation and inactivation. The phenotypic variation in men with KS could be explained by the extent to which genes escape inactivation.

Many genes on the X ch are highly expressed in testis, ovaries, and brain; thus, it is not surprising that abnormalities seen in men with KS affect brain and testis function (Wilda et al, 2000; Zechner et al, 2001; Khil et al, 2004). Although the mechanism of gene up-regulation in mammalian or human brain are not yet clear, it is believed that methylation of specific amino acids on H3 and H4, with subsequent chromatin changes, is critical to normal control of gene expression (Akhtar, 2003). Werler et al (2011) have studied the expression of selected X ch genes known to escape X ch inactivation in the mouse model and shown altered expression of these genes in male XXY mice compared with male XY mice and female XX controls. It is plausible that a similar phenomenon exists in humans. Thus, abnormal inactivation of supernumerary X chromosomes could help explain the cognitive and physical variations seen in individuals with KS.

One limitation of this study is that skewed inactivation of the supernumerary X ch in men with KS was demonstrated with the use of semiquantitative PCR only. It is important to confirm this finding with real-time quantitative PCR and multiple clinically significant X ch–specific genes. On the basis of the present results, a more detailed analysis using additional markers has already been initiated by the authors.

The question remains as to which genes inactivated on the X chromosome affect the phenotypic variation seen in men with KS. It is also important to consider that the inactivation status in this study was evaluated in the patients' peripheral leukocytes and might not reflect tissue-specific inactivation patterns. Further studies using M-PCR and DNA from specific tissues, such as the testis, might be helpful in addressing this question. Although the optimal diagnostic protocol and indications of screening for KS need to be deduced from further work, M-PCR appears to be a cost-effective and accurate test to diagnose KS in men. This method could have other potential applications in prenatal genetic diagnosis and forensic medicine.

Acknowledgments

The Internal Review Board of the Weill Cornell Medical College approved this study. The authors also thank Ms Kristin Saunders for her help in editing this manuscript.

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Trainee Page

As you are reading this issue, you should all be making plans to attend the American Society of Andrology's 30th Annual Meeting, to be held in Seattle, Wash, from March 30 to April 6, 2005. This meeting always provides the perfect opportunity to mingle with fellow trainees and to network with other andrology professionals. This issue's segment showcases Darius Paduch's own unique journey as a trainee. We encourage you to approach him and any of the other authors who have shared their experiences and wisdom in Trainee Page articles. These individuals are ideal resources and are always happy to answer any questions that you may have regarding your career goals and plans for the future.

The Journey of a Trainee

by Darius A. Paduch

Six more months is the time I still have left to reach the Holy Grail of andrology training. This is the time before I cross the Bridge that lies between the comfortable terrains occupied by the others like me (the trainees) and the ultimate, promised ''Major University'' earth inhabited by the faculty. I am finally able to see the light at the end of the tunnel. But looking back at this long journey, I don't really think about this as a tunnel—it sounds too dark. I think about the past 20 years (yes, 20) as a great adventure—and believe it or not, I would repeat it over and over again. Sure I wish that I was 25, had my 2 degrees (MD, PhD), more publications, less gray hair and more hair in general, made ''6 figures,'' and so on, but training, like everything good in life, takes time, persistence, resilience, hard work, and good mentors. As you may be able to tell, I love what I do. I like andrology. I believe in physician scientists, so what I hope to accomplish here is to share some of my experiences in an effort to ease the decision making for other people who want to become clinical andrologists and scientists.

There are a couple of ways to go about accomplishing this aim, but all of them include graduating from medical school, followed by completion of a residency in obstetrics and gynecology with a fellowship in reproductive endocrinology, a urology residency and andrology fellowship, or internal medicine with an endocrinology fellowship. I think it really pays to enter a fellowship with a strong laboratory research program to get a good understanding of molecular biology. Some of you will consider entering MD/PhD programs, which I believe is a great option if you know early in your life that science is your way of life. For me personally, entering the Polish equivalent of an MD/PhD program and struggling to finish my PhD thesis while doing a residency in urology was a great opportunity to learn the craftsmanship of biostatistics, molecular biology techniques, and designing and analyzing clinical trials.

Perhaps the most important part of this experience was that it exposed me to great mentors, without whom I would not be where I am today. I will repeat it over and over again: mentors are the most important ingredient in the recipe for success in medicine and science. Thus far, I have been very fortunate in this regard, and I will always be very grateful to the people who mentored me. Sometimes it took years to understand what they meant, but I never regret listening to their advice. One of my mentors told me: ''Well, son, I can help you to stay here, but sooner or later you need to have the guts to grab your banner and carry it into the battlefield of science and research like a brave hussar would do.'' For non-Polish readers, Poland has had a long history of hussars (soldiers on horseback) carrying a banner and going into battle against all odds. At this early point in my education, it did seem that the odds were against me. I was a foreigner dreaming of becoming a urologist and working with the best reproductive professionals in the United States. At that time, I felt very uncertain about my future, but looking back, I realize that my mentor was closing an easy pathway for me in order to push me further and further in a more challenging direction. As things turned out, his efforts were successful.

I came back to Poland and started to work hard in the lab. I designed clinical research on adolescent varicocele, did my best in medical school and in my PhD program, and studied hard to pass United States Medical Licensing Exam. It was really hard work, but it paid off. I presented an award-winning paper at the American Academy of Pediatrics meeting, and it helped me to get into urology

residency at Oregon Health Sciences University, in Oregon. This success is thanks to the guidance of my excellent mentors, Dr John M. Barry and Dr Eugene F. Fuchs. No, I am not just offering flattery—I have experienced this firsthand. You need to find good mentors very early in your career. They are essential all through medical school, in your PhD program, or in your residency. Those people will not only help you open the doors to many places (postdoc laboratories, fellowships, etc), but they will also give you a better perspective on life and what is important in science. You don't have to agree with your mentors all the time, but try to understand what they mean—it really helps.

My residency was very tough. It was before the controlled work hours for interns and residents, so I was at work all the time. The research progress was a second priority but never out of my mind—I knew that I wanted to get into a good fellowship, but without publications (and a good mentor) it would be almost impossible. After the 6 years of residency, I was getting tired (and poor) and my friends were questioning my sanity. ''Why would you now begin a 2-year fellowship, suffering through the financial hardship and waste of potential income just to have a fellowship paper?'' they asked me. There were and are plenty of jobs for urologists without fellowships. Well, I never looked at things this way. I have always wanted to be a clinician scientist, teaching and working in an academic institution. I have learned from Dr Gene Fuchs and Dr John Barry that ''money will always come,'' and that you need to do ''what is the best for you and your career.'' So at the age of 36 I moved to New York City to start a fellowship in andrology at the Weill Medical College. Age should never prevent you from reaching your dreams. This has been the best decision I have ever made. Training is about being exposed to people who can inspire you, give you new ideas, and just be there for you when you need someone to listen. The three people I have met here, Dr Peter N. Schlegel, Dr Marc Goldstein, and especially Dr Matthew Hardy, have each been all and more than I could ever have wanted from a mentor. If you don't share this type of invaluable connection with your boss, I suggest that you change the boss or move to a different institution. It is not worth your time and effort to be working in place where you aren't both challenged and inspired every day.

So what is the recipe for success? I think that it is important to have a dream and a general sense of one's life's goals. Once you make this decision, be persistent and flexible. Write, read, work hard, and don't feel jealous of your friends who are already making hefty salaries, buying houses, and starting families. Feel confident that you are different. Science can be very rewarding, despite the fact that it takes a lot of time to reach your career goals. So don't give up. There is always a light at the end of the tunnel. Nobody wants to be a PhD student forever. Although it may sometimes feel that long, have faith that you will graduate one day.

So here I am, 6 more months—was it all worth my time and the gray hair I've acquired? Yes, I think it was. I have 4 job offers from really good universities, I feel more confident about my own skills, and I have learned that as a physician scientist you become very special and unique. You will be the one to take the clinical question to your other basic science colleagues, you will be able to work on the bench answering clinically important questions, and you will be able to see a smile on your patients' faces because you were able to bridge the gap between science and the practice of medicine. I don't think that there is a better job, calling, or way of spending one's life. Although the adventure you will undertake is long, the fruit of this work is very rewarding.

Acknowledgments

Dedicated to my mother, brother, and John W C.

Klinefelter syndrome: an argument for early aggressive hormonal and fertility management

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Objective: To investigate the impact of early hormone replacement therapy (HT) on sperm retrieval rates in patients with Klinefelter syndrome (KS).

Design: A systematic review of the relevant literature using the PubMed NLM database.

Result(s): There are no randomized controlled trials evaluating the impact of HT on sperm retrieval or reproductive outcomes in men with KS. On average, surgical sperm retrieval rates in men with KS are around 51%, with a range of 28%–69%. Young patient age is the most consistent positive predictor of sperm retrieval. Lower retrieval rates have been reported in a small subset of KS adults who previously received exogenous T, although the nature, duration, and reason for such therapy in these patient subsets are unknown.

Conclusion(s): Early HT is recommended in patients with KS, but its effect on fertility potential has not been definitively studied. Larger studies are needed to better answer this question. Cryopreservation of sperm-containing semen or testicular tissue from a significant proportion of affected adolescents is possible, even when containing very low numbers of spermatozoa, and should be considered to maximize future fertility potential. (Fertil Steril® 2012;98:274–83. ©2012 by American Society for Reproductive Medicine.)

Key Words: Klinefelter syndrome, T replacement, fertility, sperm retrieval, testicular sperm extraction (TESE)

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Klinefelter syndrome (KS) is the most common chromosomal disorder in men, with an estimated prevalence of 0.2% in the general population, 3% among infertile men, and up to 11% in men with nonobstructive azoospermia (1). Yet, KS remains frequently underdiagnosed, because of the wide phenotypic variation among affected individuals, and the lack of established screening programs. Only 25% of men with KS are diagnosed during their lifetime, with fewer than 10% being diagnosed before puberty (2). The disorder is categorized by X-chromosome polysomy, with X-disomy being the most common variant (47,XXY). Characteristic features of KS include small testes, hypogonadism, and infertility (3). Higher grades of X-chromosome polysomy are associated with a more severe clinical presentation, whereas genetic mosaicism (46,XY/47,XXY) usually results in a milder phenotype (4).

Despite the wide variability in clinical presentation, all patients with KS suffer from absolute or relative hypergonadotropic hypogonadism and impaired spermatogenesis. Recently, considerable emphasis has been placed on the early diagnosis and management of KS, with emphasis on early interventions such as physical, occupational, and speech therapy, combined with hormone manipulation therapy (5, 6). At present, there is no randomized controlled trial evaluating the efficacy or outcome of this approach. The goal of T replacement therapy (TRT) in adolescents with KS is to promote linear growth, increase muscle mass, preserve bone density, and allow for the development of secondary sexual characteristics. It is believed that promoting normal physical development in adolescents with KS may have positive psychological benefits (7). Meanwhile, the impact of hormonal manipulation on the fertility potential of KS adolescents is unknown. Although T supplementation has a beneficial effect on semen volume, there is concern that the exogenous T may conversely have a detrimental impact by further suppressing testicular function (8).

Testosterone levels are usually normal in infants with KS, and they show appropriate response to gonadotropin

Received April 24, 2012; revised May 30, 2012; accepted June 1, 2012; published online June 23, 2012.
A.M. has nothing to disclose. D.A.P. has nothing to disclose.
Supported by The Frederick J. and Theresa Dow Wallace Fund of the New York Community Trust, and the Robert Dow Foundation.
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Fertility and Sterility® Vol. 98, No. 2, August 2012 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2012.06.001

stimulation during childhood (9). Most boys with KS have sufficient levels of circulating T to initiate puberty, but often fail to progress adequately. Clinically this phenomenon is described as decelerated or poor progression of puberty and is evident as poor development of facial hair, masculinization, and emotional and social development delay. Testosterone levels increase during early puberty, stabilize during midpuberty in the low-to-normal range, and then decline (10, 11). A progressive increase in LH and FSH to hypergonadotropic levels is also noted around midpuberty, with a concomitant decrease in inhibin B to undetectable levels. Due to progressive testicular steroidogenic dysfunction, exogenous T supplementation is required in mid-to-late puberty to allow for full pubertal development and age-appropriate attainment of secondary sexual characteristics.

Spermatogenic function of the testes is similarly affected. Boys with KS have lower testicular volumes during childhood compared to age-matched controls (12). Testicular growth briefly increases after the onset of puberty until midpuberty, and declines thereafter, accompanied by low serum T and elevated gonadotropin levels (13). In unaffected boys, testicular growth at puberty is the result of germ cell proliferation, with seminiferous tubules accounting for 80% of the total testicular volume. In contrast, in boys with KS, the increase in testicular volume is likely the result of interstitial cell proliferation and hyperplasia (13). Histopathological studies demonstrate that in boys with KS, germ cell differentiation is arrested at the spermatogonium or early spermatocyte stage. Spermatogonia appear to have difficulty entering meiosis, instead proceeding directly to apoptosis at the onset of puberty (14). A gradual deterioration of seminiferous tubules is seen over time, accompanied by the tubular hyalinization and Leydig cell hyperplasia that is characteristic of adult KS testes (12).

Increasingly, the onset of puberty is being recognized as the critical time to address the fertility potential of patients with KS. Using fluorescence microscopy, we have identified ejaculated sperm in semen samples from a large proportion of our adolescent patient population with KS (15). Unfortunately, sample preparation for fluorescence imaging is toxic to sperm, making it impossible to appreciate motility. However, successful pregnancies resulting from rare ejaculated sperm in the semen of men with KS have been previously reported (16, 17). Historically, men with KS were considered infertile. However, it is now well accepted that isolated foci of spermatogenesis can exist in the testes of patients with KS (18). This discovery, along with advances in assisted reproductive technologies (ART) during the past two decades, have made paternity possible for men with KS. Because puberty is associated with a progressive decline in steroidogenic and spermatogenic functions of the testes, early sperm retrieval and semen or testicular tissue cryopreservation has been recommended by several invesigators (8, 9, 19–21) for patients with KS. How the timing and success of sperm retrieval may be influenced by hormone replacement therapy (HT) in these patients is a matter of debate.

This review aims to investigate the impact of early HT on sperm retrieval rates in patients with KS. Predictors of sperm retrieval and fertility outcomes are examined, based on a comprehensive search of the published literature. Future directions for fertility preservation in patients with KS are also discussed.

MATERIALS AND METHODS

A systematic search of the National Library of Medicine PubMed database was performed, up to and including March 2012. Search terms included "Klinefelter syndrome" combined with "testosterone replacement," "testosterone supplementation," "fertility," "TESE," "ICSI," or "sperm retrieval." The search was limited to English language publications involving human subjects. To identify the most relevant publications for this review, specific exclusion criteria were sequentially applied. Only studies that involved nonmosaic patients with KS were considered. Publications that did not contain "Klinefelter syndrome" in the title or abstract were excluded. Studies in which the primary outcome measure was unrelated to reproductive outcomes, were also excluded. All remaining publications—review articles, primary research studies, irrespective of study design, and case reports—were included in the final analysis. Figure 1 outlines the selection process.

The primary outcome for this review was surgical sperm retrieval in the setting of HT.

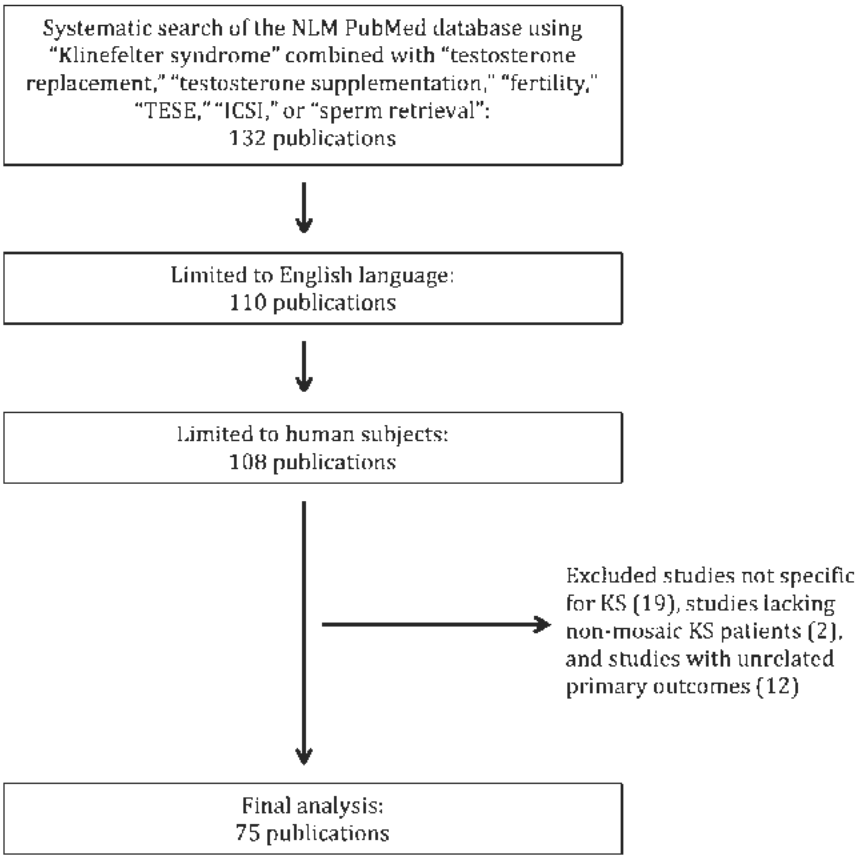
RESULTS

The literature search identified a total of 132 scientific papers. After application of the exclusion criteria, 75 publications were eligible for inclusion in the final analysis (Fig. 1). The majority of these publications were review articles addressing the management of adolescents and adults with KS, or case reports documenting paternity in patients with KS, achieved spontaneously or with ART. Sixteen studies evaluated success rates of sperm extraction, of which eight identified predictors of sperm retrieval, and only three specifically discussed the impact of previous HT on the rates of sperm retrieval. Despite the general consensus that HT should be offered to patients with KS starting in the peripubertal period, at present the number of studies published addressing the optimal timing and modality of such therapy are limited. Notably, the literature search revealed the lack of any randomized controlled trial evaluating the impact of HT on sperm retrieval or reproductive outcomes in men with KS.

Table 1 summarizes the success rates and predictors of sperm retrieval from men with KS, based on data published to date (22–37). Although the focus was on patients with nonmosaic KS, the report from Schiff et al. (37) was included because of the low proportion (7%) of mosaic KS patients and similar sperm retrieval outcomes between mosaic and nonmosaic patients. The report from Tuttelmann et al. (38) was excluded because, in their series, patients with KS could not be distinguished from non-KS patients with nonobstructive azoospermia.

Sixteen studies involving a total of 497 patients were analyzed. The average overall sperm retrieval rate was 51%, with a range of 28%–69% at various centers, using different surgical techniques. Series in which microdissection testicular sperm extraction (TESE) was used reported higher retrieval rates than series using nonmicrodissection TESE (61% vs.

FIGURE 1



Outline the study selection process.

Mehta. Aggressive management of KS. Fertil Steril 2012.

47%). Three studies reported previous use of TRT in a subset of the patients, however, only two of these specifically examined sperm retrieval rates among men who had previously been on exogenous T. Eight of 68 men in one series and 5 of 42 men in the other had received previous TRT, which was stopped at least 6 months before microdissection TESE, with sperm retrieval rates of 20%–25% (23, 37).

Although the studies varied in their conclusions as to predictors of sperm retrieval, positive predictors included young age and preoperative T levels close to or within the normal range, either at baseline or with HT (aromatase inhibitors, clomiphene citrate [CC], or hCG) (23, 29, 32, 34). Important, serum LH or FSH levels were not predictive of testicular spermatogenic function. Larger testicular volume was associated with a higher rate of sperm retrieval in the series by Madgar et al. (34), but this finding was not corroborated by other investigators. In a study by Westlander et al. (39), neither testicular Doppler ultrasonography results nor the detection of >10% mosaicism in peripheral lymphocytes or buccal tissue correlated with successful sperm recovery. Histopathological finding of rare tubules with germ cells were associated with detection of spermatozoa in the 4 of 19 patients who had successful sperm recovery in this series (39). Conversely, at our institution pretreatment testicular histology has not been found to be helpful in predicting the success of sperm retrieval; 70% of KS men with Sertoli cell–only pattern on testicular histology had sperm found on microdissection TESE (18).

DISCUSSION

Approximately 8% of adult men with KS have sperm present in the ejaculate. These men typically have cryptozoospermia or severe oligospermia, with sperm concentrations $<1 \times 10^6$/mL, and impairment in sperm motility and morphology (22). Successful pregnancies have been achieved using ejaculated sperm and ART (40). However, surgical sperm retrieval and intracytoplasmic sperm injection (ICSI) have dramatically improved the fertility potential of men with KS. Tournaye et al. (36) first reported on successful sperm retrieval in men with KS using TESE in 1996. The first pregnancies achieved using ICSI of ejaculated and testicular sperm were reported 2 years later (41). Since then, there have been a reported 101 children born to fathers with nonmosaic KS (42). This number is likely an underestimation. With the use of microdissection TESE, sperm retrieval rates in patients with KS are considered equivalent to those in men with nonobstructive azoospermia (18).

TABLE 1

Success rates and predictors of sperm retrieval in men with Klinefelter syndrome.

Reference	No. of patients[a]	Drug therapy	Procedure	Sperm retrieval rate (SRR %)	Predictors of sperm retrieval	Fertility outcome
Tournaye (1997)	15	None	TESE	8/15 (47)		Not reported
Friedler et al. (2001)	12	None	TESE	5/12 (42)		1 set of twins[c] and 2 singletons born using fresh sperm, 1 set of twins born and 1 spontaneous early abortion using frozen-thawed sperm
Levron et al. (2000)	20	None	TESE	8/20 (40)		1 set of triplets, 1 set of twins, and 2 singletons born
Yamamoto et al. (2002)	24	None	TTB[b]	12/24 (50)	Decreased levels of androgen-binding protein were a negative predictive factor	1 set of twins and 3 singletons born
Madgar et al. (2002)	20	None	TESE	9/20 (45)	No significant difference in SRR with respect to FSH or LH, higher serum T, testicular volume, and response to hCG stimulation test were positive predictive factors	Not reported
Westlander et al. (2003)	19	None	TESE	4/19 (21)	No significant difference in SRR with respect to inhibin B	2 pregnancies
Seo et al. (2004)	25	None	TESE	4/25 (16)	No significant difference in SRR with respect to age, FSH, T, or testicular volume	50% ICSI fertilization rate using fresh sperm
Vernaeve et al. (2004)	50	None	TESE	24/50 (48)	No significant difference with respect to age, testicular volume, FSH, T, FSH:LH ratio, or androgen sensitivity index	Not reported
Okada et al. (2005)	51	None	TESE	26/51 (51)	No significant difference in SRR with respect to T, LH, FSH, or testicular volume; age < 35 y was a positive predictive factor	Not reported
Okada et al. (2005)	10	None	mTESE	6/10 (60)		3 singletons born and 1 spontaneous early abortion using frozen-thawed sperm

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TABLE 1

Continued.

Reference	No. of patients[a]	Drug therapy	Procedure	Sperm retrieval rate (SRR %)	Predictors of sperm retrieval	Fertility outcome
Schiff et al. (2005)	42[d]	5/42 had received TRT (stopped at least 6 mo before mTESE); 36/42 with serum T < 300 ng/dL were treated with hCG, CC, aromatase inhibitors, or combined therapy before mTESE	mTESE	29/42 (69)	No significant difference in age with respect to SRR; prior TRT was associated with lower SRR (20%)	18 pregnancies, 21 live births
Emre Bakiricioglu et al. (2006)	74	14/74 had received TRT (stopped at least 6 mo before mTESE)	mTESE	42/74 (57)	No significant difference in SRR with respect to T, FSH, LH, or testicular volume; younger age was a positive predictive factor	Not reported
Kyono et al. (2007)	17	None	TESE	6/17 (35)	No significant difference in SRR with respect to T, FSH, LH, or testicular volume; younger age was a positive predictive factor	5 singletons born using fresh sperm; 1 set of twins and 1 singleton born using frozen sperm
Koga et al. (2007)	26	None	mTESE	13/26 (50)	No significant difference with respect to age, testicular volume, T, FSH, LH, PRL, E_2, inhibin B	Not reported
Ramasamy et al. (2009)	68	8/68 had received TRT (stopped at least 6 mo before mTESE); 56/58 with serum T < 300 ng/dL were treated with hCG, CC, or aromatase inhibitors before mTESE	mTESE	45/68 (66)	No significant difference with respect to FSH, LH, or testicular volume; age <35 y, and normal preop T and T:E ratio[e] were positive predictive factors; prior TRT was associated with lower SRR (25%)	28/68 (41%) men achieved paternity
Selice et al. (2010)	24		TESE	9/24 (38)	No significant difference with respect to age, testicular volume, FSH, LH, T, free T, E_2, SHBG, inhibin B; signs of hypoandrogenism were a negative predictive factor	Not reported

Note: CC clomiphene citrate; ICSI intracytoplasmic sperm injection; mTESE microdissection testicular sperm extraction; preop preoperative; SHBG sex hormone-binding globulin; SRR sperm retrieval rate; TESE testicular sperm extraction; TTB therapeutic testicular biopsy; TRT testosterone replacement therapy.

[a] Reflects the number of patients with nonmosaic Klinefelter syndrome included in each study.

[b] Therapeutic testis biopsy.

[c] Triplet pregnancy reduced to twin pregnancy.

[d] 39 (93%) had nonmosaic Klinefelter syndrome, 3 (7%) were mosaic 46,XY/47,XXY. Unable to distinguish mosaic patients for exclusion. Overall outcomes reported.

[e] T to E_2 ratio.

Mehta. Aggressive management of KS. Fertil Steril 2012.

A higher pregnancy rate (PR) after ICSI has been reported with the use of freshly retrieved sperm (27, 37). However, cryopreserved sperm have been successfully used by several groups (28, 29, 35), obviating the need for repeat surgical procedures for sperm retrieval. Sperm cryopreservation is a challenge when the sperm retrieved are limited in number or quality, but improvements in technology may render this approach more feasible and successful in the future.

With the evidence available to date, it is difficult to ascertain the impact of TRT in men with KS. It is unknown whether the suppressive effect of exogenous T on testicular steroidogenic and spermatogenic function is fully reversible in, and if so, for what period of time. In the reports by Ramasamy et al. (23) and Schiff et al. (37), the total number of adult men who were on TRT was limited to 12. The duration of therapy in these patients is unknown. The reason for initiation of TRT may have been severe testicular dysfunction at baseline, which would not be expected to improve after cessation of exogenous T, even with additional medical therapy. The route of administration of TRT in this subset of patients is also unknown, and may have variably impacted the degree and duration of suppression of testicular function (43). Conversely, at present, we have performed microdissection TESE in three adolescents with KS aged 12–15 years, treated with topical T gel and aromatase inhibitors for more than 6 months up to and including the date of surgery, and successfully retrieved rare sperm for cryopreservation in two of these patients (Paduch DA, unpublished data). Whether concomitant exogenous T at the time of surgery impacted the number or quality of sperm retrieved is unknown. For all of these reasons, although intuitive, it is difficult to reliably attribute the low sperm retrieval rates in these 12 patients to the previous use of TRT alone. Larger studies are required to better answer this question.

Testosterone Deficiency and HT

There are three physiological peaks of serum T during normal male development. The first occurs in the prenatal period, the second, termed minipuberty, occurs during the first 2–4 months of life, and the third occurs at adolescence. It has been questioned whether T deficiency occurs during all three times in males with KS, and consequently, whether androgen therapy should be considered when activation of the pituitary-gonadal axis first occurs. Several investigators have documented a peak in serum T in infants with KS around 3 months of age. Although two series reported lower mean serum T levels in infants with KS versus controls (44, 45), other studies have shown normal or high normal T levels in infants with KS (46, 47). In addition, children with KS have normal levels of T, FSH, LH, and inhibin B in the prepubertal period, along with a normal serum T response to hCG stimulation (10). Thus, there appears to be no indisputable evidence of hypoandrogenism in infants and prepubertal children with KS.

Recently, it has been postulated that androgen receptor CAGn length polymorphisms may contribute to phenotypic variance in patients with KS (48). Penile length is a sensitive indicator of androgenization, and an inverse correlation between CAGn repeat length and penile length has been noted (49). According to expert opinion, penile length is boys with KS is often less than 46,XY boys, but not in the range of micropenis (50). Nevertheless, some physicians choose to treat infants and children with KS for decreased penile length. Due to lack of alternative dosing regimens, treatment regimens are often borrowed from the long-standing protocols of T therapy for micropenis (50). There are only anecdotal reports of androgen therapy during infancy leading to improved outcomes in patients with KS. Additional studies are certainly needed to determine whether androgen therapy should be considered on the basis on CAGn repeat length in males with KS.

On the basis of review articles considering HT in boys with KS, current practice recommendations include initiation of therapy in early-to-midpuberty, or at the onset of hypogonadism (7, 9, 50–52), to ensure the normal timing of completion of puberty and prevent the symptoms and sequelae of long-term androgen deficiency. The single observational study on the topic found quality of life to be reduced in patients with KS on various forms of TRT (53). Data on serum T levels was not provided in this questionnaire-based study, and quality of life scores were better in KS patients with higher education levels, indicating that the findings may be reflective of the underlying diagnosis of KS (6) more so than a consequence of TRT.

There are no specific guidelines as to the optimal modality of TRT for patients with KS. Age-appropriate formulations and dosage may be extrapolated from the Endocrine Society's Clinical Practice Guidelines for the treatment of hypogonadal men (54). In our andrology practice, which includes more than 200 patients with KS, we typically initiate TRT after the onset of puberty using topical T gel, which appropriately and adequately increases serum T levels in the majority of patients, and avoids anxiety and needle phobia associated with IM T injections. Compliance with daily gel application can be challenging for some adolescents, but is helped by caregiver supervision and involvement. Intramuscular TRT nevertheless remains a popular modality for TRT, especially for patients seeking alternatives to transdermal applications (9, 50). One case report in the literature has suggested the use of implantable T pellets as a viable treatment option in noncompliant adolescents with KS (55). Because patients with KS can also have elevated serum E_2 levels or an elevated E_2-to-T ratio, we also use aromatase inhibitors in adolescents with gynecomastia or central obesity, particularly when response to T gel is poor. Longitudinal therapeutic outcomes in our cohort of patients are currently being evaluated for publication in the near future.

Impaired Spermatogenesis

The etiology of testicular degeneration in KS is not well understood. Increased expression of genes on the supernumerary X chromosome, intratesticular hormonal imbalance, defects in spermatogonial stem cells, and abnormal apoptotic activity of Sertoli and Leydig cells have all been proposed as possible underlying factors (21). There is some evidence to suggest that the degenerative process may begin during fetal

or neonatal life (52). Mikamo et al. (56) showed a progressive decline in the number of spermatogonia from 24%–0.1% of control values during the first year of life in infants with KS, although other quantitative studies have shown normal germ cell counts (52, 57). Diminished number or complete absence of spermatogonia has likewise been reported in prepubertal boys with KS, with normal-appearing Sertoli and Leydig cells (58). Immunohistochemical studies demonstrate that in early adolescence, the majority of boys with KS have germ cells in their testes (13, 14). The number of spermatogonia, especially adult dark spermatogonia, is, however, markedly reduced. The onset of puberty is associated with accelerated and progressive depletion of these germ cells, which may precede elevation in serum gonadotropin levels (13, 14).

In the setting of KS, immature Sertoli cells are incapable of transforming into mature adult Sertoli cells during puberty (13). Compared to healthy males, a smaller proportion of Sertoli cells in males with KS express androgen receptors (14). Furthermore, these receptors are aberrantly located in the cell cytoplasm rather than on the cell surface (14). Some investigators have suggested that the decline in inhibin B levels during mid and late puberty is reflective of the loss of Sertoli cell number and function (7). Sertoli cell secretory dysfunction has been associated with unsuccessful attempts at testicular sperm retrieval (25). Whether impaired spermatogenesis in the 47,XXY testis is intrinsic to germ cells, or due to the inability of Sertoli cells to support normal germ cell development is not fully resolved at present. Likewise, Leydig cell failure (impaired steroidogenesis) may be intrinsic to Leydig cells, or due to germ cell depletion, Sertoli cell dysfunction, on elevated intratesticular E_2 levels. Further studies are required to elucidate the molecular mechanisms involved.

Germ cell differentiation into mature spermatozoa is arrested in the testes of boys with KS. Based on testicular biopsies from 14 adolescents, Wikström et al. (13) determined that maturational arrest occurs early at the level of type A spermatogonia, before meiotic division. Other investgators, however, have reported meiotic arrest later, at the primary spermatocyte or spermatid stages, with rare foci of normal spermatogenesis (1, 7, 59). This distinction clearly has an impact on fertility outcomes with ART, and will likely gain importance as future advances are made in the area of in vitro sperm maturation.

Early Sperm Retrieval

That younger age is a positive predictor of sperm retrieval in men with KS is not surprising, as the decline in testicular function secondary to KS is progressive, beginning in puberty and worsening during adulthood (60). In the studies included in the review, the majority of sperm retrieval procedures were performed in adults who presented for fertility treatment. It is reasonable to postulate that sperm retrieval during adolescence may have led to even better sperm retrieval rates in these patients. Damani et al. (20) have reported successful cryopreservation of sperm-containing testis tissue in a 15-year-old adolescent boy with KS. Using selective DNA fluorochrome and fluorescent microscopy, we have found sperm present in 70% of ejaculated semen samples from adolescents with KS aged 12–20 years of age (Mehta et al., Poster 53, American Society of Andrology Meeting 2012). In our practice, we routinely recommend semen cryopreservation in adolescents who have sperm present in the ejaculate. Our limited experience, to date, with surgical sperm retrieval in three adolescents with KS has also been positive.

Testicular dissection for sperm extraction has known negative effects on testicular function, with a temporary decline in serum T that recovers 12–18 months postoperatively (61). Irreversible testicular atrophy and hypogonadism is much less common. One report of outcomes in men with KS who underwent conventional or microdissection TESE showed postoperative decline in serum T with no improvement after 12 months (62). In a second report, T recovered to 50% of baseline in men with KS 12 months after microdissection TESE (63). Although these results may reflect preexisting testicular dysfunction in the study population that worsened postoperatively, these reports did not account for the wide range of T levels among the subjects, or the possibility of natural decline in T over time in men with KS. No postoperative effect on serum T was noted after testicular tissue extraction in the adolescent with KS (20).

In the absence of substantial data assessing the impact of HT on sperm retrieval rates (SRR) in patients with KS, we recommend sperm retrieval and cryopreservation as early as possible, and, when feasible, before the initiation of exogenous T therapy. Early to midpuberty may be the best time to consider sperm retrieval, when there is a brief increase in testicular size and serum hormone concentrations are relatively within the normal range. The use of non-T–based HTs, such as hCG, CC, and aromatase inhibitors, which theoretically stimulate testicular steroidogenesis, may be considered before planned surgical sperm extraction in hypogonadal patients. The decision to use these therapies should be made on an individual basis.

Genetics Risks to Offspring

In a questionnaire-based survey of patients with KS, 90% of respondents expressed a desire to father children (64). However, 70% of them also identified safety concerns with the use of TESE-ICSI, in terms of chromosomal, congenital, or developmental abnormalities in their offspring (64). The majority of offspring born to men with KS have been healthy, with a normal 46,XX or 46,XY chromosomal complement (18). However, the conception of 47,XXY pregnancies has certainly been reported, resulting in spontaneous early abortion or elective termination (65, 66).

An increased rate of sex and autosomal chromosomal aneuploidy has been reported in sperm from men with KS (24, 67–70). Whether this increased rate is specific to KS offspring, or reflective of the rate seen in ICSI offspring in general, is unknown. The largest series on this topic was published by Levron et al. (24), who showed chromosomal abnormalities in 7 of 112 (6.3%) patients, of which five were related to sex chromosomes and two to chromosome 18. Two different theories have been put forth to explain the higher rate of aneuploidy in sperm from KS males (71). Either the testis is populated with 47,XXY germ cells that

are able to complete meiosis and produce hyperhaploid spermatozoa, or rare foci of 46,XY germ cells are present in the testis, which are susceptible to meiotic errors due to the abnormal testicular environment, thereby resulting in hyperhaploid sperm. Evidence in support of the first theory comes from studies where similar chromosomal patterns were seen in 47,XXY Sertoli cells, spermatogonia, and primary spermatocytes, as in hyperhaploid secondary spermatocytes, spermatids, and spermatozoa, reflecting a common 47,XXY origin (1, 24, 25, 68). Conversely, other studies have shown that although Sertoli cells maintain a 47,XXY karyotype, germ cell lines in the KS testis are 46,XY, leading to euploid meiotic spermatocytes and normal haploid gametes (59, 72). Data from mouse models show that donor XY germ cells can develop into haploid germ cells in the XXY environment, implying that the testicular environment may be less important than the chromosomal complement of the germ cell line (73).

Given the potential for increased chromosomal abnormalities in the offspring of men with KS, preimplantation genetic diagnosis of embryos obtained using TESE-ICSI has been recommended. Staessen et al. (70) have argued that without preimplantation genetic diagnosis, the chance of selecting and transferring normal embryos for implantation is high, because even embryos with normal morphology may have underlying abnormalities. In their series, the rate of normal embryos for KS couples was significantly lower than that for normal controls (54% vs. 77.2%) (70). However, preimplantation genetic diagnosis is not routinely used by many centers, either due to lack of availability, or because the majority of offspring of KS couples are normal (33). Indeed, KS fathers using ART have fewer than theoretically expected XY or XX disomic sperm and embryos. This suggests either a strong selection against disomic sperm, or that only haploid sperm are able to fertilize an ovum and lead to pregnancy. The latter would explain the relatively favorable birth outcomes. Nevertheless, the higher rate of chromosomal abnormalities detected in preimplantation embryos remains a concern. The use of preimplantation genetic diagnosis in KS couples undergoing ICSI should, therefore, be considered.

Future Directions

Until advances in reproductive medicine allow us to accurately predict fertility outcomes for individuals with KS, nonazoospermic patients identified at puberty or before they are ready for fatherhood should be counseled with regard to sperm cryopreservation. The cryopreservation of testicular tissue containing mature spermatozoa is a suitable alternative for severely oligospermic or cryptozoospermic patients (74). Naturally, legal, ethical, and risk-benefit considerations must all be taken into account, particularly when pursuing sperm cryopreservation in minors. Informed consent must be obtained from parents, with appropriate assent from minors (8).

Cryopreservation of testicular tissue should also be considered in younger adolescents who have spermatogonia present in the seminiferous tubules, in the absence of more differentiated cell types (75). Recent studies show that human testicular tissue can be cultured for several weeks without essential loss of spermatogonia (76). Early results also suggest that spermatogenesis can take place under culture conditions, yielding normal spermatids with some fertilization potential (77). With the current scientific focus on in vitro maturation of spermatogonia, it is reasonable to hope that such technology would be available for clinical use in the next one or two decades. Certainly, cryopreservation of testicular tissue to be used for evolving technologies, such as spermatogonial maturation ex vivo, is experimental at present, and should be done under a research protocol.

In conclusion, early hormone substitution therapy is recommended in the patients with KS to complete normal pubertal development and prevent the adverse consequences of hypogonadism. At present, the benefit, if any, of T supplementation during the first 2–3 months of life is unclear. Initiation of TRT should instead be considered after the onset of puberty, when serum T levels being to decline. Randomized, placebo-controlled trials showing the impact of T substitution on fertility outcomes in patients with KS are lacking. But the potential benefits of T replacement in young men with KS nevertheless must be balanced with concerns for the maintenance of fertility potential. Cryopreservation of semen samples from boys with KS in early puberty is possible, even when containing very low numbers of spermatozoa, and should be offered to appropriate patients before initiating T supplementation. Surgical sperm retrieval should be considered for fertility preservation in adolescents who are unable to provide a semen sample, or who are azoospermic, after an informed discussion with the patient and parents.

Despite limited testicular volume, extensive tubular sclerosis, and markedly elevated FSH levels, sperm retrieval with TESE and microdissection TESE is possible for 50%–70% of men with nonmosaic KS, based on results from different centers. These surgical sperm retrieval rates, along with ICSI, have remarkably improved the ability of individuals with KS to father children.

At the present time, the option for fertility preservation in boys with KS includes the possibility of in vitro maturation of immature germ cells or spermatogonia into mature spermatozoa or, at least, elongated spermatids capable of fertilizing ova using ART. Increasing literature support and clinical experience with KS patients both strongly support implementation of fertility preservation measures in adolescent boys with KS.

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RBM Online - Vol 10. No 6. 2005 747 754 Reproductive BioMedicine Online; www.rbmonline.com/Article/1732 on web 19 April 2005

Article

Novel mutations in testis-specific ubiquitin protease 26 gene may cause male infertility and hypogonadism

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Abstract

Patients ($n = 188$) with non-obstructive azoospermia (NOA), and 17 fertile controls were screened for sequence changes in the ubiquitin specific protease (*USP*) 26 gene. Semen analysis, hormonal evaluation, and testicular biopsies were performed. DNA was extracted from whole blood. Denaturing high-performance liquid chromatography was used to screen for single nucleotide polymorphisms. Amino acid sequences were determined in samples with mutations. Twenty out of 188 (10.6%) infertile men had amino acid changes in USP26. No changes were found in fertile controls. 1090C→T substitution and (363insACA; 494T→C; 1423C→T) change were found in 3.3 and 1.9% of patients respectively. Serum testosterone concentrations and testicular volume were lower in the mutation positive group compared with the non-mutation group (272 versus 366 ng/dl; $P = 0.01$) (volume: 7.88 versus 10 ml, $P = 0.03$). Six out of 28 (21%) patients with Sertoli cell-only syndrome, and two out of 18 (11%) patients with maturation arrest had mutations in the *USP26* gene. There were no live deliveries in couples with the USP26+ mutation, and three live deliveries in the group without mutations. The *USP26* gene may be of importance in male reproduction. Mutations in this gene may be associated with male infertility, and may negatively affect testicular function.

Keywords: *infertility, ubiquitin, USP26*

Introduction

Infertility affects one in 10 couples, with 30 50% of couples suffering from male factor infertility. Although varicocoele, history of undescended testis, Klinefelter syndrome, infections and drugs can account for 70 80% of male infertility, the aetiology of idiopathic male infertility is often unknown. Growing evidence suggests that genetic defects affecting spermatogenesis may be responsible for many cases of idiopathic infertility (Huynh *et al.*, 2002). Although it is known that chromosomal aberrations such as Klinefelter syndrome (KS), and Y chromosome microdeletion are associated with infertility, the molecular mechanisms responsible are not known (Lanfranco *et al.*, 2004). Recently reported, testis specific-genes located on the X chromosome, revealed an array of candidate genes for male infertility (Wang *et al.*, 2001). One of those genes is ubiquitin specific protease 26 (USP26), located on the X chromosome, at Xq26.2. Ubiquitination and deubiquitination of macromolecules regulates the cell cycle, chromosomal structure, vacuolization, and gene silencing (Glickman *et al.*, 2002). Deubiquitination of macromolecules by deubiquitinating enzymes (DUB), including ubiquitin proteases, can rescue macromolecules from degradation through substrate-specific, N-terminal-dependent, enzymatic reaction (Wilkinson, 1997; Wing, 2003). Because of the importance of DUB in cell cycle regulation, as well as testis-specific expression of this gene, it was decided to choose USP26 as a novel candidate gene for the study of male

infertility. It has previously been reported that preliminary data indicates increased number of mutations in the *USP26* gene in men with severe male factor infertility (Paduch *et al.*, 2004). The initial observations have been further strengthened by a recent report describing a 363insACA in USP26, which was identified in 9.5% of patients with Sertoli cell-only syndrome (SCO; Stouffs *et al.*, 2004). The present study provides the first extended phenotypic description of patients with mutations in USP26 and evidence linking mutations in USP26 to male infertility.

Materials and methods

Patient selection

This study is part of a larger, IRB approved, study of novel genes in male infertility. A DNA repository with over 1500 DNA samples obtained from patients referred for Y microdeletion screening has been established. Two hundred and thirteen randomly chosen patients with azoospermia or severe oligozoospermia who were referred for genetic Y microdeletion screening were included in the current project. Seventeen fertile men served as a control group. Patients with known chromosomal aberrations, or Y chromosome microdeletions were excluded from this analysis. The results of semen analysis, serum hormones (T, FSH, LH, E), karyotype and physical examinations were obtained from patients' records. Some, but not all, patients underwent miscrosurgical testicular sperm extraction (TESE), and for those patients intraoperative findings, results of intracytoplasmic sperm injection (ISCI), and final pathology of testicular biopsies were available. Testicular volume was measured by an attending physician using an orchidometer.

Mutation analysis

DNA from all subjects in this project was extracted using the Stratagene DNA extraction kit (200600) (Stratagene, La Jolla, CA, USA) and stored at 20°C. USP26 sequence (AF285593) was obtained from the NCBI web site http://www.ncbi.nlm.nih.gov (date of accession: 7/10/2003). The *USP26* gene has 2794 bp and no introns, and it encodes a 913 amino acid long globular protein. The entire gene was divided into six overlapping fragments (spans: 94 to 2869) ranging from 409 to 600 bp in length. Each fragment was amplified in a single polymerase chain reaction (PCR) using a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). The primers used are listed in **Table 1**. Genomic DNA 150 ng was added to the PCR mixture containing 2.5 IU of Transgenomic Optimase polymerase (Transgenomic, Omaha, NE, USA), 1.5 mmol/l of $MgSO_4$, 0.5 µmol/l of each forward and reverse primer, 200 µmol/l of each dNTP (deoxynucleoside triphosphate), 5 µl of reaction buffer (10× stock solution), and water up to 50 µl. Annealing temperatures were 2°C higher then calculated primer melting temperatures, as recommended by the Transgenomic Optimase product insert. Amplification was carried for 35 cycles.

The presence of PCR product was verified by a high performance liquid chromatography system (HPLC; Wave Nucleic Acid Fragment Analysis System Model 3500A; Transgenomic). PCR product 5 µl was injected into the HPLC column. Concentration of the acetonitrile buffer and elution temperature was calculated for each amplicon, and controlled with Wavemaker software (Transgenomic) (**Table 1**). The chromatograms were stored on a hard drive. The presence of product was defined as a chromatogram peak above 2.5 mV. If the peak was lower than 2.5 mV, the PCR reaction was repeated or 15 µl of product was injected. Wild-type amplicon from fertile men was hybridized with each PCR product at 95°C, and slowly cooled down in a thermocycler. Hybridized samples were again injected into the HPLC column, and chromatograms were stored on a hard drive. The presence of sequence change in the examined sample was evident by the presence of two to four peaks on its chromatogram, whereas samples with no sequence changes had only one peak. Amplicons from samples with sequence changes were

Table 1. Primers used to amplify the overlapping fragments of ubiquitin specific protease (*USP*) 26 gene, length of amplicon product and temperatures of high performance liquid chromatography screening used for each amplicon. bp = base pair.

USP26 fragment no.	*Fragment size bp*	*Primers*	*Temperatures of screening (°C)*
1	600	For: 5′-ACCAATACTAGAAATAGGATCATTCTG-3′; Rev: 5′-TCCCACTTCCTTTTGCTATCTC-3′	55.1, 55.4, 57.5
2	526	For: 5′-GCACAACACAGAAGGAAATCAA-3′; Rev: 5′-CCGTGGCATATTTTCTCTGG-3′	56.0, 56.8
3	593	For: 5′-CGGTTACACAAAGTGGGATAAA-3′; Rev: 5′-TTCTTTGGGGAAGGTTGATG-3′	52.0, 55.0, 56.4
4	554	For: 5′-TGTTGCACTCCATTGCTTGT-3′; Rev: 5′-TTGCTGCTGTCTTCTGCTTG-3′	56.0, 56.8
5	409	For: 5′-TTAAAGGGGCAAGCAGAAGA-3′; Rev: 5′-TGAGGGGCTTGTTCACAGAT-3′	55.6, 57.2, 58.9
6	569	For: 5′-ATCTGTGAACAAGCCCCTCA-3′; Rev: 5′-CCATGGAGGAAGTGGTATCG-3′	55.8, 57.9

collected using the Transgenomic fragment collector, sequenced with forward and reverse primers, and results of sequencing were compared with wild-type sequence. Nucleotides were counted using A of ATG start codon as +1 nucleotide.

Reverse transcriptase (RT)–PCR

In order to examine USP26 mRNA expression patterns in human testis, total RNA was extracted from nine testicular samples: three patients with normal histology, three patients with maturation arrest, and three patients with SCO, using RNA STAT-60 (Tel-Test, Freindswood, TX, USA). The quality of RNA extraction was verified by denaturing gel electrophoresis, using standard protocol with formaldehyde and MOPS. Two distinct ribosomal RNA bands were identified in each sample examined. Using the Titan One Tube RT-PCR system (Roche Diagnostic, Indianapolis, IN, USA), a cDNA library was generated from 1 µg of total RNA, at 50°C for 30 min, followed by PCR according to the manufacturer's instructions. To amplify USP26 from cDNA, the following set of primers was used: 5′-GCACAACACAGAAGGAAATCAA-3′ and 5′-CCGTGGCATATTTTCTCTGG-3′ (526-bp long product). Oestrogen receptor alpha, which is expressed in testis, was used to verify the quality of cDNA synthesis and PCR reaction. To exclude genomic amplification, PCR was performed with the same total RNA samples without reverse transcriptase. Products were analysed on 4% agarose gel. Reactions were performed in triplicate to ensure consistent results.

Statistical analysis

Student's *t*-test was used to compare means between groups, except for comparison of testicular volumes, where Mann Whitney *U*-test was used. Fisher exact test was used to calculate statistical significance of assisted reproduction and sperm retrieval rates between groups with and without mutation.

Results

Phenotypic changes identified in patients with USP26 mutations

Sequence changes with amino acid changes or insertion were found in 20 (10.64%) out of 188 patients screened (**Tables 2** and **3**). For detailed discussion of identified mutations, please see below.

Results of testis biopsies were available in 49 patients screened for mutation. Twenty-one per cent (six out of 28) of patients with SCO had mutations in the *USP26* gene, and 11% (two out of 18) patients with maturation arrest had mutations in the *USP26* gene. No mutations were found in three patients with hypospermatogenesis (**Tables 3** and **4**).

To answer the question whether mutations in the *USP26* gene are functionally relevant, results of semen analysis, testicular size and hormonal evaluation were compared in 20 patients with mutations to 168 patients without mutations. Patients with KS and deleted in azoospermia factor (AZF) a, b or c deletions were excluded from the analysis. Only patients seen at Weill Medical College had data available for verification.

Review of available semen analyses showed that all patients with USP26 mutation were azoospermic, whereas only 66% of patients without mutations were azoospermic.

Mean testosterone concentration in patients with mutation in USP26 was lower by 104 ng/dl. This difference was statistically significant ($P = 0.01$) (**Table 5**). There were no statistically significant differences in concentrations of FSH, LH, or oestradiol between groups with and without mutation (**Table 5**).

Patients with the *USP26* gene mutation had a statistically significant difference in testicular size, as compared with patients without the mutation (7.88 ml, SD = 4.5 ml versus 10 ml, SD = 4.41 ml; $P = 0.03$) (**Figure 1**).

Forty-two patients out of 188 screened underwent TESE with ICSI for male factor infertility. In this group, seven patients had the USP26 mutation. Although not statistically significant, patients with this mutation had lower sperm retrieval rate two out of seven patients (29%) versus 15 out of 35 patients (43%). There was one pregnancy and no delivery in the group with mutation, and seven pregnancies and three live deliveries in patients without the mutation. It remains to be seen if those differences will become statistically significant with an increased number of patients screened.

Detailed description of identified mutations

Seven patients had 1090C→T substitution, with leucine to phenylalanine amino acid change in position 364 (L364F). Another patient had 1090C→T and 1274C→T substitutions in the same allele, causing leucine to phenylalanine amino acid change in position 364 (L364F), and proline to leucine change in position 425 (P425L). 1090C→T mutation seems functionally significant, since two patients in this group had SCO (**Table 3**).

Four patients had characteristic insertion of 363insACA in nucleotide position 363, causing a threonine insertion in amino acid position 121, followed by tandem of 494T→C and 1423C→T substitution, with amino acid sequence changes: leucine into serine, and histidine into tyrosine respectively (363insACA; 494T→C; 1423C→T). One patient had insertion of ACA in nucleotide position 363, causing a threonine insertion in position 121, followed by tandem of 494T→C without 1423C→T substitution (363insACA; 494T→C). One patient had 1423C→T substitution without any changes. It is interesting to note that the ACA insertion followed by 494T→C and 1423C→T substitution occurs in the same allele, properly described as (363insACA; 494T→C; 1423C→T). It seems that 363insACA together with 494T→C by itself can negatively affect function of the USP26, as evident by low testosterone in patient no. 15 bearing this mutation (**Table 3**). At the same time, biopsy of the patient with the sole 1423C→T mutation revealed SCO. This patient also had very low testosterone. There was no difference in testosterone concentration among four patients with the (363insACA; 494T→C; 1423C→T) sequence variant and the nine other mutations identified.

Table 2. Frequency and type of detected mutations among 188 patients with azoospermia and severe oligozoospermia.

Nucleotide change, amino acid position	*No. patients*	*Percentage*
1090C→T, L364F	7	3.72
363insACA, T121ins and 494T→C, L165S and 1423C→T, H475Y	4	2.13
1737G→A, M579I	2	1.06
1737G→A, M579I and 2202A→C, K734N	1	0.53
363insACA, T121ins and 494T→C, L165S	1	0.53
1090C→T, L364F and 1274C→T, P425L	1	0.53
1037T→A, L346H	1	0.53
1423C→T, H475Y	1	0.53
1497G→A, E500K	1	0.53
1976C→T, T659M	1	0.53
Totals	20	10.64

Table 3. Details of identified mutations in ubiquitin specific protease 26 gene. SCO = Sertoli cell-only syndrome, MA = maturation arrest, Te = testosterone, E = oestradiol, T-L = left testicular volume (ml), T-R = right testicular volume (ml). Amino acids: C = cysteine, L = leucine, E = glutamate, M = methionine, F = phenylalanine, N = asparagine, H = histidine, P = proline, I = isoleucine, S = serine, K = lysine, T = threonine, Y = tyrosine. Yq: deletion of distal arm of Y chromosome was verified by microdeletion screening and not karyotype.

Patient no.	*Histology*	*FRG1*	*FRG2*	*FRG3*	*FRG4*	*FRG5*	*FRG6*	*Sperm count*	*Te[a]*	*FSH[a]*	*E[a]*	*LH[a]*	*T-L*	*T-R*	*Karyotype*
1				1037 T→A, L346H											
2	SCO				1737 G→A, M5791	2202 A→C, K734N		0	197	20.3	21	11			46XY
3				1090 C→T, L364F											
4	SCO			1090 C→T, L364F and 1274 C→T, P425L				0	280	35.2	32	11	2	3	46XY
5				1090 C→T, L364F											
6	SCO				1423 C→T, H475Y			0	163	15.4	90	818	6	4	46XY
7	SCO	363ins ACA Ti2lins	494, T→C, L165S		1423 C→T, H475Y			0	346	11	28		14	14	46XY
8				1737 G→A, M5791				0	270	56	30	23			46XY
9		363ins ACA, Ti2iins	494 T→C, L165S	1423 C→T H475Y											Yq

continued on page 751

Table 3. *continued*

Patient no.	*Histology*	*FRG1*	*FRG2*	*FRG3*	*FRG4*	*FRG5*	*FRG6*	*Sperm count*	*Te*[a]	*FSH*[a]	*E*[a]	*LH*[a]	*T-L*	*T-R*	*Karyotype*
10				1090 C→T, L364F				0	472	24.6	33	6.9	12	12	46XY
11						1976 C→T, T659M									
12				1090 C→T, L364F				0	210	4.3	20	4.4			46XY
13		363ins ACA, T121ins	494 T→C, L165S					0	266	23.4	19	8.3	5	6	46XY
14	MA	363ins ACA, T121ins	494 T→C, L165S		1423 C→T H475Y			0	278	43	9		2	2	46XY
15	MA				1737 G→A, M5791			0	371	31.4	49		12		46XY
16				1090 C→T, L364F											
17				1090 C→T, L364F											
18	SCO			1090 C→T, L364F				0	220	6.3		5.1			
19					1497 G→A E500K			0	281	2.7	42	2.6	8	10	46XY
20	SCO	363ins ACA, Tl2lins	494 T→C, L165S		1423 C→T H475Y			0	189	4.9	17		10	12	

[a]Units as shown in Table 5.

Table 4. Results of mutation screening in patients with (USP26+) and without (USP26) identified mutations in ubiquitin specific protease (*USP*) 26 gene who underwent testicular sperm extraction.

Histology	*USP26+ (%)*	*USP26 (%)*	*Total*
SCO	6 (21)	22 (79)	28
Maturation arrest	2 (11)	16 (89)	18
Hypospermatogenesis	0	3 (100)	3
Total	8 (16)	41 (84)	49

Table 5. Hormonal characteristics of patients with (USP26+) and without (USP26) identified mutations in ubiquitin specific protease 26 gene.

Category	*No.*	*Testosterone (ng/dl)*		*FSH (IU/l)*		*LH (IU/l)*		*Oestradiol (ng/l)*	
		Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
USP26+	13	272	84	21.42	16.4	9.50	5.8	33.54	21.9
USP26	64	366	139	18.14	12.0	8.19	5.0	29.43	20.0
P-value		0.01		NS		NS		NS	

NS = not significant.

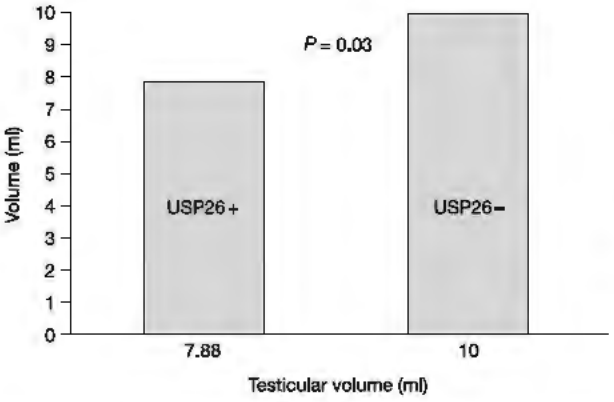

Figure 1. Patients with mutations in ubiquitin specific protease (USP) 26 (USP26+) have lower testicular volume than patients without mutation (USP26).

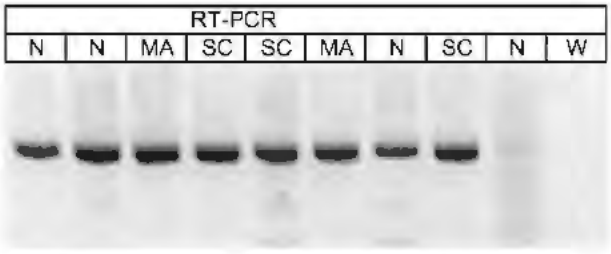

Figure 2. Results of reverse transcriptase-polymerase chain reaction (RT-PCR) with total RNA extracted from testicular samples. N = normal histology, MA = maturation arrest, SC = Sertoli cell-only syndrome, W = water.

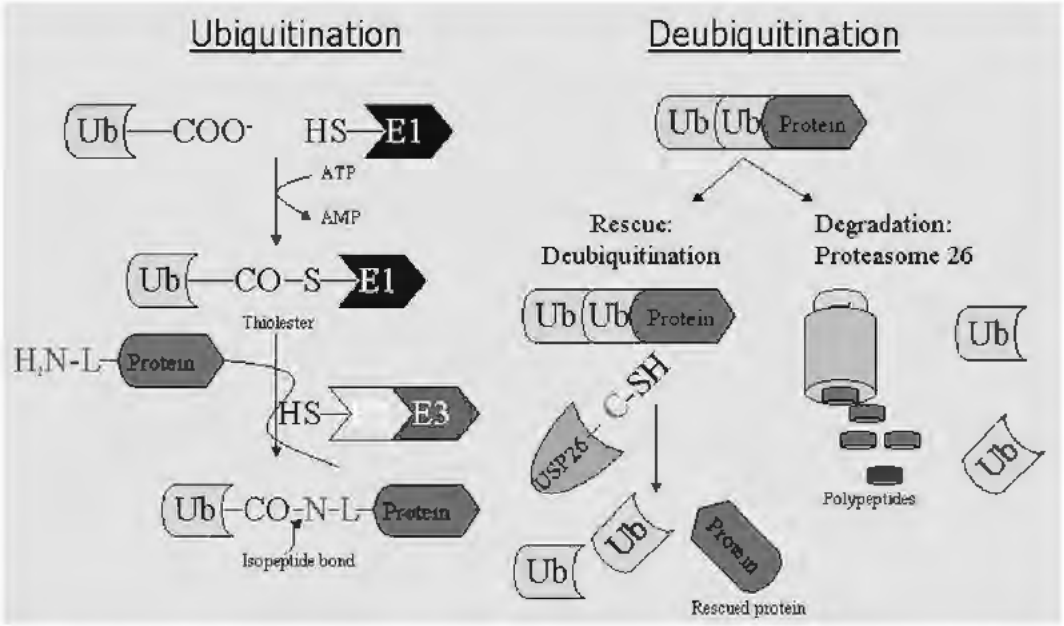

Figure 3. Schematic diagram of ubiquitination and deubiquitination pathways. Ubiquitin specific protease (USP) 26 is able to rescue proteins condemned to deactivation by proteasome system. Adequate balance between ubiquitination and deubiquitination of cell cycle and apoptotic proteins may ultimately be responsible for the fate of the cell. Ub = ubiquitin. E1 = ubiquitin-activating enzyme E1; E2 = ubiquitin-conjugating enzyme E2; E3 = ubiquitin-protein ligase E3.

Three patients had nucleotide substitution 1737G→A, with amino acid change methionine to isoleucine (M579I); however, one of the patients in this group had the additional amino acid change lysine to asparagine (K734N), as a result of 2202A→C substitution (1737G→A; 1737G→A).

The following four substitutions occurred only once in the studied population: 1037T→A, resulting in leucine to histidine change (L346H), 1423C→T with amino acid change histidine to tyrosine (H475Y), 1497G→A substitution causing glutamate to lysine change (E500K), and 1976C→T substitution with threonine to methionine change (T659M).

RT–PCR

It is thought that USP26 mRNA should be absent in SCO syndrome; however, initial data reveal that USP26 is expressed in testis with normal histology, as well in testis samples with SCO and MA (**Figure 2**). This experiment was repeated three times and each time the same pattern of expression was achieved. More experiments using real time RT-PCR, northern blot, and in-situ hybridization to better assess spatial expression of USP26 are being performed in the laboratory. Since it was shown that mutations in USP26 may cause hypogonadism, it will be important to understand how and if those mutations affect function of Leydig cells.

Discussion

This study presents the first evidence that USP26 may be important in male infertility and testicular dysfunction. USP26 belongs to a large family of DUB. Ubiquitination and deubiquitination of macromolecules regulates the cell cycle, chromosomal structure, vacuolization, and gene silencing (Wilkinson, 1997; Wing, 2003; Ciechanover *et al*., 2004) During the cell cycle, molecules important for apoptosis or cell proliferation can be turned off by tagging a particular molecule with ubiquitin (Ub) (Zhang *et al*., 2004) (**Figure 3**). This molecule will then undergo degradation by the proteosome system. Deubiqutination of macromolecules by DUB, including ubiquitin proteases, can rescue macromolecules from degradation through substrate-specific, N-terminal-dependent, enzymatic reaction. DUB identified so far are tissue specific with high specificity for substrate. DUB have cysteine and two histidines consensus patterns. Cysteine consensus pattern: G-(LIVMFY)-x(1,3)-(AGC)-(NASM)-x-C-(FYW)-(LIVMFC)-(NST)-(SACV)-x-(LIVMS)-Q (C is the putative active site residue) and histidine consensus pattern: Y-x-L-x-(SAG)-(LIVMFT)-x(2)-H-x-G-x(4,5)-G-H-Y (The two H's are putative active site residues) (Wilkinson, 1997). Structure analysis revealed that none of the identified mutations are located within active sites; however, the majority of mutations are found within the DUB substrate recognition site. Hence, it is postulated that the activity of deubiquitination in the poliUb assay test may not be affected in mutation-specific functional analysis of USP26, but identified mutations may affect the interaction between the substrate and the USP26.

USP26 was first identified by Wang *et al*., and it is believed to be expressed only in testis. Recently, Stouffs *et al*. reported data on mutation analysis in the *USP26* gene in patients with infertility (Stouffs *et al*., 2005). Their manuscript reports one of several mutations also reported by the present authors (Paduch *et al*., 2004) during the AUA meeting in 2004. Both this paper and Stouffs *et al*. reported characteristic insertion of 363insACA in nucleotide position 363 (363insACA; 494T→C; 1423C→T) causing a threonine insertion in position 121, followed by tandem of 494T→C and 1423C→T substitution, with amino acid sequence changes: leucine into serine, and histidine into tyrosine respectively. Although all eight patients in the report of Stouffs *et al*. had combined haploallelic change following 363ACAins, in the present study, one patient was found with only 363insACA and 1423C→T (363insACA; 494T→C), and one patient with 1423C→T as a sole mutation. It will have to be determined from further studies which of those three mutations in one allele affect function of USP26 protein. In the current group of patients, nine new mutations were identified other than (363insACA; 494T→C; 1423C→T) substitution (**Table 3**). Although all patients with Klinefelter syndrome and AZF a, b or c deletion were excluded, one patient with (363insACA; 494T→C; 1423C→T) change had Y chromosome distal arm microdeletion. It is interesting that Stouffs *et al*. noticed the same association in one of their patients. It is unknown if (363insACA; 494T→C; 1423C→T) substitution plays any role in pathogenesis of Yq microdeletion or if the above finding is a pure coincidence.

The differences in the total number of mutations identified in the screened population between the present report and the one by Stouffs *et al*. (2005) can be explained by the fact that Stouffs *et al*. screened the entire gene in only 42 patients with SCO, whereas the present study screened 214 patients and then excluded patients with KS and AZF a, b or c deletion. Other than 1090C→T substitution and (363insACA; 494T→C; 1423C→T) change which occurred in 3.3 and 1.9% all the other new mutations identified by us occurred in less than 1% of patients (0.94 0.47%); therefore, to detect those changes, one needs to screen the entire gene in over 100 patients.

It is likely that the identified mutations have functional significance of sincethe same type of mutation was found by two groups in diverse populations in Europe (Stouffs *et al*.) and in the USA (present study). It is important to notice that more fertile controls were so that statistical analysis of the frequency of mutations could be performed. It is astonishing that the frequency of (363insACA; 494T→C; 1423C→T) substitution in patients with known histology is almost exactly the same in the present report 4/44 (9.1%) and that published previously (9.5%).

This study is the first to report that the presence of mutations in USP26 is associated with significantly lower testicular volume, and lower testosterone concentration. Patients with USP26 mutations have mean testosterone concentration below the low normal range (300 ng/dl) in this study. No study so far has evaluated spatial localization of the USP26 mRNA or protein, hence it is possible that either the expression of USP26 is limited to germ cells and testicular dysfunction is secondary to aplasia of germinal epithelium, or alternatively USP26 may be expressed in Leydig or Sertoli cells as well. However, it has been established that patients with idiopathic infertility often suffer from hypogonadism. (Andersson *et al*., 2004). Preliminary data showed that USP26 is expressed abundantly in patients with normal histology, SCO, and

maturation arrest. Limited expression of USP26 was anticipated in patients with SCO, but experiments so far have proved us wrong. It is likely that in humans expression of USP26 is not limited to germ cells. This issue is currently being evaluated. Regardless of the mechanism, the data provide early evidence that USP26 mutations may cause testicular dysfunction.

In the present series, 21% of patients with SCO and 11% of patients with MA had mutation in USP26. Stoufts *et al.* reported 7.2% rate of mutation in USP26 among patients with SCO, and no mutation in MA; however, their paper only reported one type of mutation (363insACA; 494T→C; 1423C→T). Since four patients with the same mutation were identified and two had SCO, the actual frequency of 363ACAins mutation among men with SCO is almost the same: 7.7% (2/44).

Forty-two patients underwent TESE, and the results of sperm retrieval as well as pregnancy rates are lower in the group with USP26 mutation; however, the differences are not statistically significant at this point. More assisted reproduction data are being collected to further analyse the role of USP26 mutations in outcomes of assisted reproduction.

In conclusion, these data indicate that mutations in USP26 may cause significant testicular dysfunction and male infertility, and may potentially affect the outcomes of assisted reproduction. It is hoped that this report will stimulate further research into deubiquitiuation and its effect on male fertility.

Acknowledgements

This work was supported by the Frederick J and Theresa Dow Wallace Fund of the New York Community Trust. Internal Review Board of the Weill Medical College of Cornell University approved this study.

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Received 3 February 2005; refereed 17 February 2005; accepted 2 March 2005.

Chapter 12

Obesity and sexual dysfunction in men

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Prevalence of obesity has been rising over the last several decades in the industrialized countries, and according to a study, 68% of US adults are overweight and 35% are obese [1]. WHO data indicate that worldwide 41 million children and 1.9 billion adults aging 18 years or older are affected by obesity. Obesity affects every aspect of daily life, reproduction, and health in general.

Obesity has been linked to increased risk of diabetes mellitus (DM); hypertension (HTN); and risk of cardiovascular events, such as stroke, cancers, and osteoarthritis. As normal sexual function is a part of normal reproductive system, health consequences of obesity on low testosterone and erectile dysfunction (ED) are becoming of significant clinical importance.

Diagnosis of obesity is based on body mass index (BMI). BMI is calculated by dividing weight by square of height. BMI is very useful and has been extensively used in epidemiological studies but should not be used as a sole measure of obesity as it does not measure per se the percentage of total body fat. Using WHO criteria for adults older than 20, normal BMI is between 18.5 and 25; subjects are overweight if their BMI is between 25 and 30, they are obese if their BMI is 30−40, and morbidly obese if BMI > 40.

Physiology of sexual function

The normal erectile function depends on intact neurovascular function of the penis—arterial blood flow and venous closing mechanism, normal sensory innervation of the penis, normal central nervous system processing and integration of sexual cues in brain and spinal cord, and intact autonomous nervous system responses (Fig. 12.1).

Sexual cues (tactile, auditory, olfactory, visual, and recollection of past experience) are integrated into the hypothalamic and cortical centers and descending signals are sent through autonomic nervous and spinal cord to thoracic and lumbosacral regions of sexual response

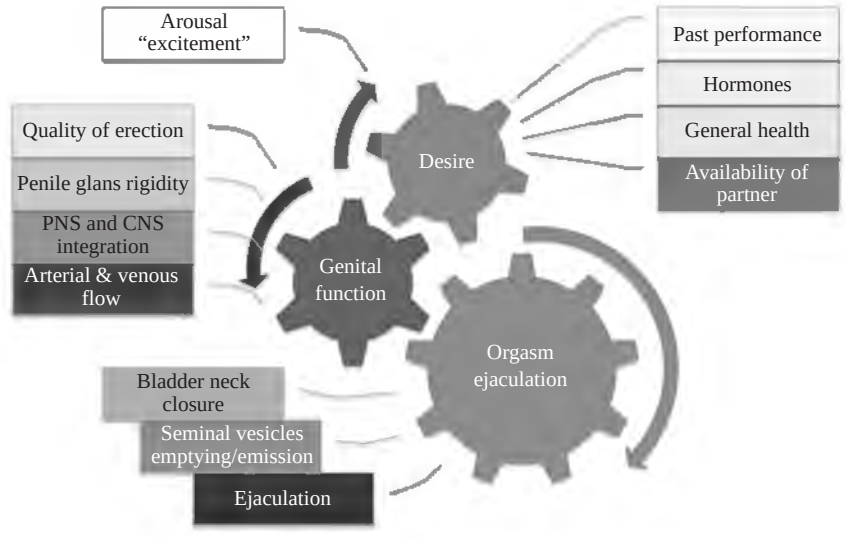

FIGURE 12.1 Elements of normal sexual response.

Obesity and Gynecology. DOI: https://doi.org/10.1016/B978-0-12-817919-2.00012-7

(Fig. 12.2). Somatosensory input from penis is carried through dorsal nerve of the penis through pudendal nerve and ends in the sacral region S2−S4. Posterocentral gyrus in cortex represents primary penile sensory activation. Scrotum and pubic area are innervated by the branches of ilioinguinal (L1) and femoral cutaneous nerves (L2−L3) (Figs. 12.3 and 12.4). DM can impair normal conduction in pudendal and sensory nerves and has been postulated as one of the reasons for diabetic ED [2].

The descending signals reach penile erectile tissues through cavernous nerves carrying parasympathetic and sympathetic fibers (Fig. 12.4). Parasympathetic stimulation results in relaxation of smooth muscles within penis, opening of bilateral cavernosal arteries, and sudden increase in blood flow through penis, which results in tumescence (penile erection). At the same time, subtunical veins that normally drain blood from penis get closed (Fig. 12.5).

Any pathology that impedes the muscle relaxation with an increase in penile blood flow will result in ED.

Over the last two decades, most of the researches have focused on ED; however, it is well known that the presence of rigid erection is not sufficient for satisfactory reproductive and sexual performance, thus both evaluation and therapeutic interventions need to embrace different aspects of sexuality.

Sexual Dysfunction

Sexual dysfunction can be divided into ED (problems sustaining erections adequate for penetration), disorder of sex

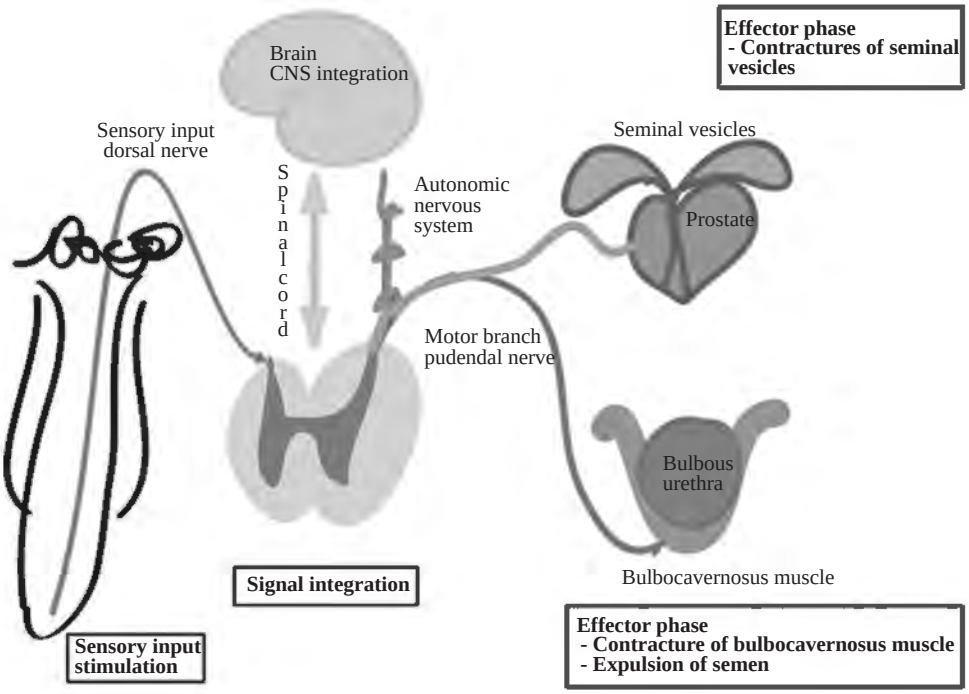

FIGURE 12.2 Neural pathways for sexual function.

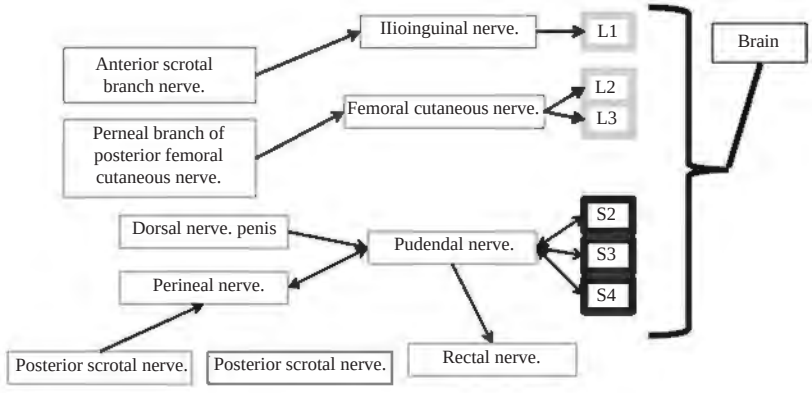

FIGURE 12.3 Sensory and motor innervation of penis and scrotum.

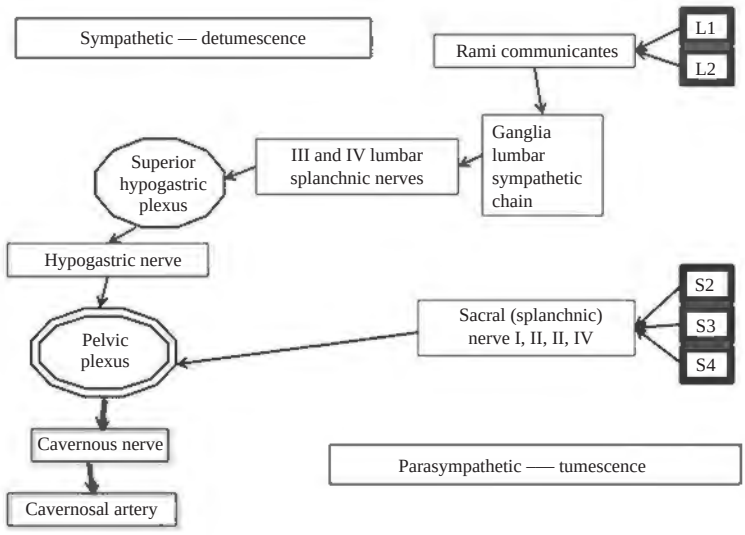

FIGURE 12.4 Sympathetic and parasympathetic innervation of penis.

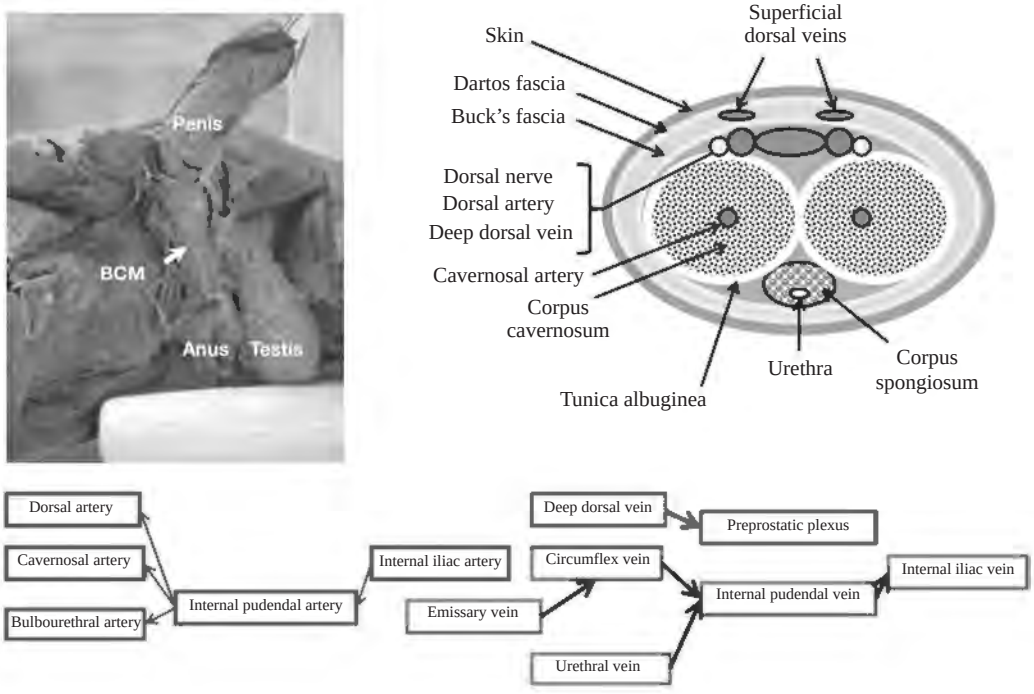

FIGURE 12.5 Vascular anatomy of penis.

drive (libido), disorder of arousal (excitement), and disorders of ejaculation and orgasm. The ejaculatory dysfunction can present itself as premature ejaculation (intravaginal latency time is less than 2 minutes), delayed ejaculation (subjective prolonged time to ejaculate), and anejaculation (lack of ejaculation). Lack of ejaculation can be the result of retrograde ejaculation (semen goes back into bladder) or lack of emission. Orgasm is a subjective sensation of enhanced pleasure followed by postcoital refractory period. Orgasm is typically associated with ejaculation in normal subjects. Orgasmic dysfunction can range from lack of orgasm through decreased sensation of orgasm (Fig. 12.6). Patients and health professionals often use the words "orgasm" and "ejaculation" as synonyms even though biologically the two phenomena are regulated by two different neurobiological pathways.

Sex Drive

Sex drive is a complex neuropsychological phenomenon affected by hormones, energy status, health, social norms,

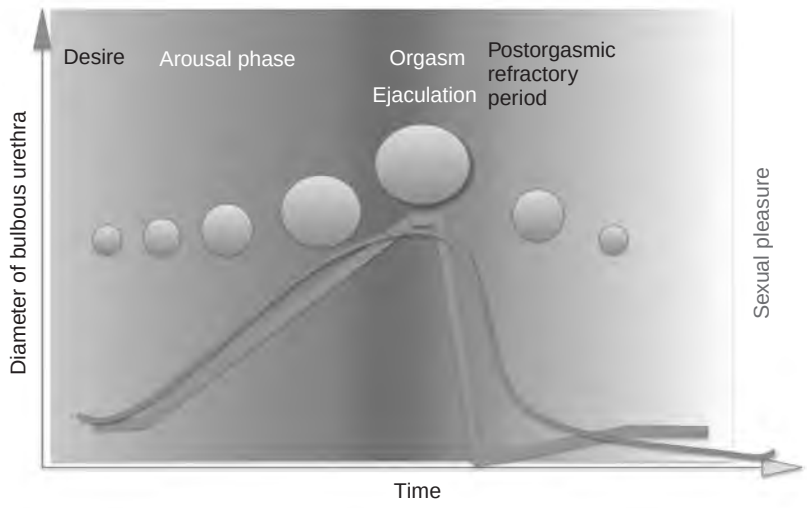

FIGURE 12.6 Phases of normal sexual response in man.

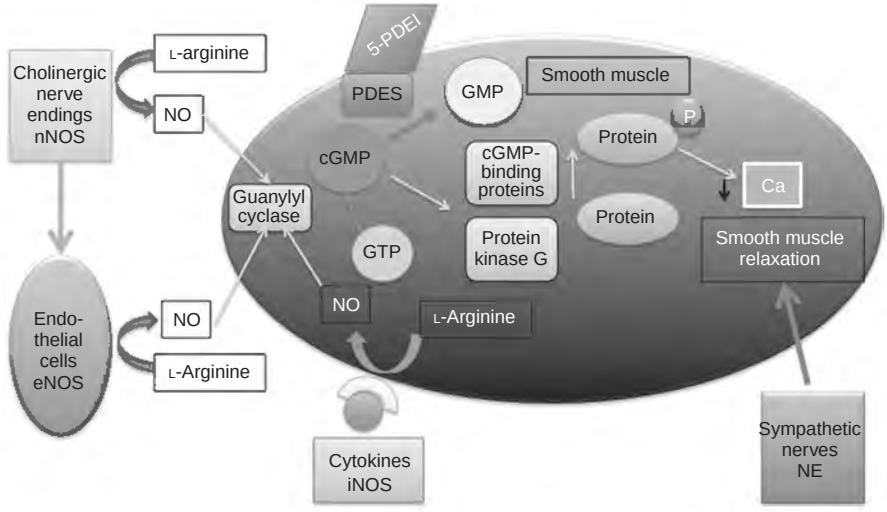

FIGURE 12.7 Molecular mechanism of erection.

and emotional well-being. Body image and recollection of positive sexual experience play important roles in sex drive and normal sexual response in both men and women. Obesity with change in body image, decrease in functional length of penis because of pubic fat pad, and limitation in physical capabilities may further erode one's confidence in sexual performance and have detrimental effect on sex drive leading to withdrawal from interpersonal relationships. Pain from osteoarthritis in knees as a consequence of obesity further limits the physical stamina leading further complicating sexual activities. Pain medications are known to lower testosterone level. Hence, consequences of obesity on body mechanics and function often result in vicious circle with weight gain—depression—functional restrains—leading to sedentary lifestyle—and further increase in weight.

Erection (tumescence) starts with stimulation parasympathetic nerves with release of acetylcholine and activation of nitric oxide synthase within nerve endings (nNOS) and endothelial nitric oxide synthase (eNOS). NOS converts L-arginine into nitric oxide—a potent vasodilator that is transported by diffusion to smooth muscles. NO activates guanylate (guanylyl) cyclase converting GTP to active cGMP. cGMP activates phosphorylation of target proteins resulting in decrease in intracellular calcium, smooth muscle relaxation, and erection (Fig. 12.7). Sympathetic nervous system and local factors such as endothelin-1 (ET-1) oppose smooth muscle relaxation and result in detumescence.

Obesity related sexual dysfunction

Obesity is known to affect hormonal levels, specifically increase in circulating estradiol (E2) level and decrease in total testosterone (TT) level. In addition, obesity-related medical comorbidities such as HTN, dyslipemia, and DM are linked to ED.

Exact mechanism of obesity-related sexual dysfunction is an area of intense research and multifactorial model with local impairment of penile tissue relaxation with global endocrinopathy and metabolic changes in nerve signaling seem to best describe clinically observed sexual dysfunction in obese men [3,4].

In rodents that are fed high-fat diet (HFD) versus standard diet, there was a clinically and statistically significant drop in cavernous strip relaxation after induction with acetylcholine (Ach) [5]. This and other studies showed a decreased concentration of cGMP in penile tissue from obese mice as well as decrease in endothelial and acetylcholine and nonadrenergic−noncholinergic nitrergic signaling thus impairing the relaxation of penis, which is necessary for normal tumescence [6]. The tissues from obese penis are much more sensitive to adrenergic stimulation and ET-1 [7]. Thus obesity decreases penile ability to relax smooth muscles and increases sensitivity to stress-related signaling impairing the balance between tumescence and detumescence, which is necessary for normal erection.

Hyperglycemia decreases NO production by eNOS by O-linked glycosylation of eNOS at the Akt target S1177 [8]. Diabetes is clearly associated with diminished endothelial production of NO [9,10].

Sexual response in men is initiated by the desire to have sexual activity. Desire is a complex neurobiological prenomen that is first experienced with initiation of puberty, thus sex steroids have always been considered a critical component of normal sexual desire. Although testosterone level and desire do not have linear correlation, multiple studies in diverse ethnical groups showed a similar association between low testosterone and low sexual desire [11−13].

It is well established that in obese men the sexual desire is negatively correlated with total and free testosterone [14]. Gastric bypass surgery improved testosterone levels and improved sexual desire.

The negative effects of obesity on sexual desire are most likely related to low testosterone and elevated estradiol, but fear of failure, changed body image, and functional limitations may be significant contributing factors.

Testosterone is produced in testes under control of luteinizing hormone (LH) and local paracrine control. Testosterone is metabolized to estradiol (E2) by aromatase CYP19 expressed in testes and abdominal fat (Fig. 12.8). Elevated levels of E2 have been found in most obese men. E2 suppresses not only central release of LH but it also has negative effects on Leydig cell function and normal T production thus explaining high prevalence of low testosterone in men with hyperestrogenism [15,16]. Elevated estradiol level in men is also associated with decrease in sperm production.

Normal sexual function depends on adequate balance between testosterone that in men should be high and estradiol that needs to be low. Treatment with aromatase inhibitors may help increasing testosterone level in some obese men with low T and elevated E2 (see next).

During sexual activity—progressive and linear increase in level of excitement—arousal is necessary to achieve orgasm and sustain erection. Little is known about effect of obesity on arousal in men, but obesity severely impairs arousal in females [17,18]. As arousal is central neurobiological process, it is very plausible that obese men suffer from similar issues with arousal. Here again, low testosterone, hormonal abnormalities, physical

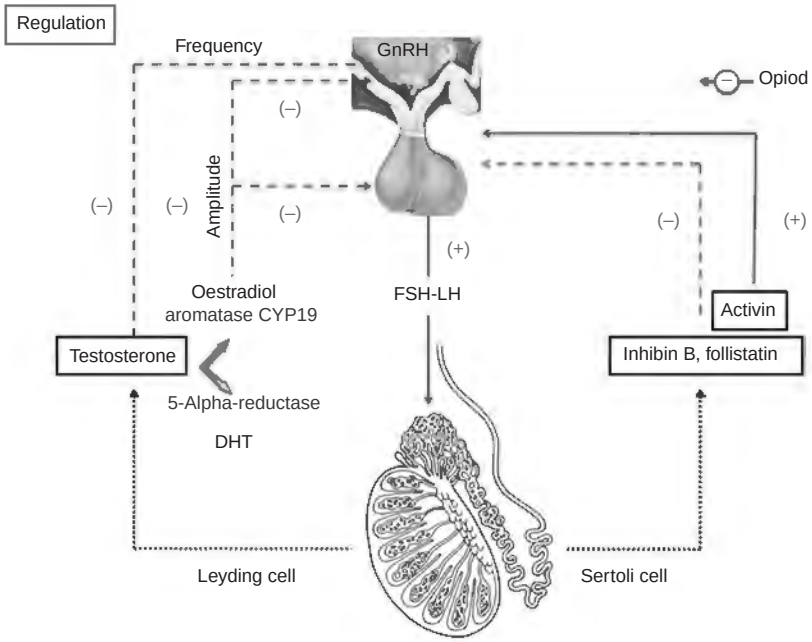

FIGURE 12.8 Continuous tonic inhibition of peripheral steroids.

limitations, and body image may interfere with normal processing of sexual cues necessary to achieve normal progression of arousal.

Sexual dysfunction and obesity-related comorbidities

It is often difficult to dissect pure effects of obesity from associated obesity-related medical comorbidities such as HTN, peripheral vascular disease, hypercholesterolemia, and diabetes.

Based on our clinical practice, we use the following functional theorem of obesity-related sexual dysfunction: (1) genital effects (relative shortening of the penis), (2) body habitus limitations (decreased accessibility to vagina due to abdominal obesity), (3) positional restrictions (due to excessive weight on partner), (4) poor muscular stamina (strength and time), (5) poor cardiovascular and pulmonary fitness (shortness of breath, poor exercise tolerance), (6) joint dysfunction due to obesity (physical limitations and pain), (7) obesity-related neuroendocrine effects (hypogonadism and hyperestrogenism), (8) obesity-related chronic inflammation cascade, (9) poor body self-image, (10) perceived perception of one's attractiveness in eyes of partner, (11) partner's concern for possible negative events during sexual activity, (12) partner's actual perception of obese male attractiveness, (13) anxiety-related to past failures in sexual domain, (14) depression either primary or reactive, and (15) metabolic consequences of obesity on overall health (HTN, DM, cardiovascular disease, etc.).

We find it useful in practice to go over different, often interconnected, dimensions how obesity relates to sexual desire and performance. Most of the published researches focused on metabolic effects of obesity but discussing with the couple's physical aspects of sexual performance and understanding specific wants and needs within couple dynamics are critically important aspects of care of patients.

Hypercholesterolemia—defined as elevated total cholesterol and low-density lipoprotein (LDL)—has been linked to increased risk of CAD in Framingham Heart Study and many others [19]. Massachusetts Male Aging Study (MMAS) showed that ED is inversely related to baseline high-density lipoprotein and prevalence of ED doubled over 9 years in men who were obese at baseline as compared to men who were not obese at baseline or follow-up [20,21]. Obesity increases risk of vasculogenic ED as assessed by penile Doppler ultrasonography [22]. But from published literature, it appears that it is a long-term obesity with obesity-related comorbidities, which correlates with ED rather than elevated BMI by itself. In Rancho Bernardo Study, which followed men over 25 years the age, hypercholesterolemia and obesity were independent predictors of severity of ED in logit model, but obesity was not an independent predictor of the presence of ED by itself.

Small nonrandomized clinical trial showed that reduction in total cholesterol to below 200 mg/dL and LDL to less than 120 mg/dL had positive effect on ED after 3.7 months of treatment [23]. However, randomized double-blinded trial (STED TRAIL) of simvastatin failed to show improvement in erectile function by simvastatin as compared to placebo. It is possible that statins have role in men with ED and hypercholesterolemia who fail to respond to sildenafil [24,25]. However, a recent study of tadalafil 20 mg three times a week versus 10 mg atorvastatin showed that tadalafil was better in restoring ED than atorvastatin, whereas atorvastatin showed some improvement in erectile function especially in men with hypercholesterolemia [26,27]. French Pharmacovigilance System Database study showed that statins may actually induce or worsen ED [28]. So far, no conclusive evidence supports the routine use of statins in patients with ED without a clear indication for the use of statins because of cardiovascular protective effects [29].

Rather conflicting results of statin treatments on improving ED in men with hypercholesterolemia may be due to short follow-up and severity of peripheral vascular disease. Reduction in prevalence of cardiovascular events with statins has been showed after 5 years of therapy by most studies (CAPS, CARE, LIPID), and there is some evidence that with longer treatment the positive effects of statin treatment on ED may be noticeable [30,31]. Another complicating factor may be lowering of TT in some men taking statins [32–34]. Thus testosterone level should be monitored in men on statins therapy. Randomized clinical trial of simvastatin 80 mg or placebo showed decrease in bioavailable testosterone by 10%. Bioavailable testosterone best correlates with biological activity of testosterone; hence, one may assume that at least in some men the drop in bioavailable testosterone may be clinically significant. In summary, it is clear that dyslipidemia is a risk factor for ED but not clear if obesity by itself without its sequel has a similar detrimental effect on ED. Often ignored but important for patient is muscle ache associated with statin therapy. Obese men have physical limitations already which can be further exacerbated by muscle aches and pains. In general, men withdraw from sex if they are in pain or discomfort. The consensus statement from AHA on statin safety indicates that 10% of men discontinue statin therapy because of muscle ache without laboratory evidence of elevation in creatinine kinase. Most of the patients will not restart statins despite normal laboratory evaluation—thus managing expectations and side effects of statins in men early on

may help with compliance. Clearly further studies are needed.

Hypertension

Approximately 50%–70% of men with HTN report varied degree of ED [35,36]. The underlying mechanism of ED in HTN seems to be HTN-induced peripheral vascular disease, and severity of ED in HTN men is correlated with the duration of HTN [37]. HTN impairs neurogenic-induced smooth muscle relaxation and reduction in superoxide dismutase in animal models [38].

Treatment of HTN with some of medications may further worsen ED and contribute to poor compliance with antihypertensive medications [39,40]. Nonselective beta-blockers, hydrochlorothiazide, spironolactone, and angiotensin II antagonists, are known to result in ED in significant number of patients thus angiotensin converting enzyme inhibitors, whereas selective beta-blockers are a better choice for men with HTN and preexisting ED [41,42]. In men who require multidrug therapy for HTN, adding 5-phosphodiesterase inhibitor improves compliance with antihypertensive therapy and results in better blood pressure control [43].

As HTN and hypercholesterolemia often occur together, most of the obese men are both antihypertensive and cholesterol-lowering agents.

Large randomized (2153 men) clinical trial of effect of rosuvastatin and candesartan plus hydrochlorothiazide versus placebo showed no difference in erectile function after 5.8 years of follow-up as measured by IIEF-EF score. Hence, clinicians have to stress on the importance of controlling cholesterol and blood pressure as a way to prevent cardiovascular events, but there is no clear evidence that the use statins or blood pressure medications can improve ED per se.

Peripheral vascular disease

Classic epidemiological study by Blumentals showed that men with ED have increased risk of peripheral vascular disease by 75% [44]. Even after adjusting for the presence of other risk factors for stroke, men with ED have a higher risk of stroke and lower risk of stroke-free survival over 5-year follow-up study in Taiwan [45]. Often ED is the first sign of peripheral vascular disease prior to claudication, thus men with ED should be screened for PVD [46]. It is believed that endothelial dysfunction combined with hypercholesterolemia is responsible for PVD and vasculogenic ED. Large epidemiological studies showed that PVD is prevalent in obesity [47]. In our practice, men with obesity and vascular ED are referred for cardiovascular evaluation and risk assessment.

Coronary artery disease

Intracavernosal (IC) arteries in penis are less than 1 mm in diameter, thus it is no surprise that often ED is first manifestation and predates coronary artery disease [48,49]. It has been shown that the presence of ED is an additional cardiovascular risk, which should be considered in stratification assessment and decision-making for need for further invasive coronary evaluation [50]. Men with ED have a higher volume of coronary calcifications as compared to men without ED [51]. The presence of ED has been demonstrated to be an independent predictor and risk factor for cardiovascular events, cardiovascular-related morality, and all-cause mortality, thus obese patients with ED should be evaluated by cardiologists to determine if they need stress test or further testing to assess their cardiovascular risks. It is our practice that in obese men with multiple risk factors for cardiovascular disease who have vascular ED, we do not initiate treatment for ED unless a patient is seen by a cardiologist, as for many men, erectile function seems to be more important than general health. The role of urologists, general practitioners, and endocrinologists in the evaluation of cardiovascular risk in men with ED is evolving, but there is no question of significant opportunity to improve one's general health.

Diabetes mellitus

Effects of DM on ED is multifactorial but neurogenic dysfunction as well as vasculopathy with impairments of NO signaling decrease in vasodilation are best understood at this point.

DM can affect sensory signaling and result in autonomic nervous system dysfunction. In obese diabetic subjects the peripheral neuropathy in lower extremities assessed by increased vibratory threshold is strongly associated with ED [52]. This should not be a surprise that tactile stimulation is one of the most important sexual cues. Micro- and macrovascular disease has been linked to DM using standardized instruments to measure sexual function [53]. Similar changes in microvascular environment with decrease in endothelial function within penis were found in animal models of DM [6].

Evaluation—General considerations

Evaluation of sexual dysfunction in obese males should include detailed medical, social, family, and sexual history combined with physical examination and laboratory testing.

Sexual history should focus how changes in BMI affect sexual drive and performance, both sexual activities with partner and alone, penetrative and other forms of sexual

activities and mechanics should also be taken into consideration. Body habitus disproportion between partners has to be assessed in culturally sensitive way. Cognitive–affective aspects of BMI and body image, self-acceptance and attractiveness, have been most exclusively studied in females, but it is established that dissatisfaction with body image is similar in men (59.5%) and among women (55.2%).

One has to consider cultural and ethnical differences in BMI and their effects on health. The concept of BMI-related metabolic transition toward HTN, hypercholesterolemia, and diabetes is affected by ethnicity. Ethnic groups with lower BMI have a higher rate of metabolic transition (Asians) compared to ethnic group with historically high BMI (Whites). This is important when advising patients about their overall risks of developing obesity-related health consequences. Practitioners should avoid projecting their own stereotypes of what is desirable and acceptable when it comes to body size and build.

Based on the high prevalence of low testosterone and sexual dysfunction in obese men, it is prudent to measure testosterone level in most obese men, especially those with loss of morning erections, decreased sex drive, fatigue, and ED. Morning total sex hormone globulin levels, and free testosterone should be obtained using the most reliable and sensitive method. In our practice, we use liquid chromatography and mass spectrometry (LC–MS) to measure TT. LC–MS has been recommended as the preferred method to measure testosterone in hypogondal men because of its lower coefficient of variance as compared to other methods [54].

FDA uses cutoff point of 300 ng/dL to establish hypogonadism in pharmacological studies. In Europe, 10.2 nmol/L is often used. One needs to understand that testosterone levels change over age, and 300 ng/dL cutoff point is probably most appropriate in older men >65 as most of the studies on testosterone level were done in older men. Free testosterone and bioavailable testosterone are useful in men with normal or normal low TT who present with symptoms. In men who have low TT, FSH, LH, PRL, thyroid profile, estradiol, and cortisol as well as baseline PSA should be obtained. CBC and liver function tests should be obtained at baseline.

In clearly hypogondal men who have elevated PRL or unexplained low or low normal LH and FSH, one may consider CT or MRI of brain with attention to pituitary and hypothalamus to exclude pituitary or hypothalamic mass.

However, obesity is associated with hypogonadotropic hypogonadism because of elevated estradiol, thus decision to obtain additional imaging studies has to be considered on individual basis.

In our practice, we also obtain ultrasensitive CRP and HgA1c at baseline in obese men who present with sexual dysfunction. In men with type I DM in the Diabetes Control and Complications Trial the risk of ED was correlated with HgA1c and men who had tight control of DM with insulin had a significantly lower rate of ED [55]. Severity of ED increases dramatically in men whose HgA1c is above 8% and with DM type II of 6 or more years [56]. Considering that a significant number of men with early diabetes are not aware of their abnormal sugars, it is critical to establish diagnosis of DM early to prevent microvascular sequels that with time may be difficult to reverse or control.

Erectile and endothelial dysfunction may have similar pathways through impaired nitric oxide activity; in obese men the endothelial dysfunction may be further impaired through increase in interleukins (IL-6, IL-8, IL-18) and CRP. Obese men with ED but not obese men without ED had a significant increase in CRP and inflammatory procytokines [57].

Elevated CRP is associated with increased risk of cardiovascular events, and in many men the presence of such objectively measured risk may aid in behavioral modifications to lose weight and to exercise.

This study also points to the fact that it is not obesity itself that results in the ED but associated vasculopathy and proinflammatory response. It is not known at this point why some obese men do not suffer from ED. Possible explanation may be limitation in BMI as marker of obesity, ethnical differences in effect of BMI on metabolic profile, and further studies with better assessment of lean body mass and percent of body fat may help us to understand the link between obesity and ED better.

We strongly advocate active screening for sexual dysfunction among obese and overweight men as it has been shown that correction of ED with medical therapy may have positive impact on glycemic control through better adherence to medical therapy and diet.

Evaluation of sexual function

Depending on practice, one can consider using screening questionnaires to assess ED. Questionnaires that assess broad aspects of sexuality specifically sex drive, orgasmic, and ejaculatory dysfunction in addition to ED may be better suited for obese men considering that they often suffer from complex sexual dysfunction when ED is only one of the domains. In our practice, we use MSHQ during initial visit and follow-up, but each physician has to choose the instrument which is most appropriate to his or her patient population and cultural and social norms.

Although BMI is most commonly used measure of obesity, we use lean body mass and percentage body fat as well as waist circumference to better stratify metabolic risks of higher BMI. Ethnical differences in BMI norms may be

considered. BMI is very useful in large epidemiological studies but may be less useful in managing individual patients.

Typically in obese patient with ED by history and verified by questionnaires other than hormonal evaluation, no further workup is needed especially if testosterone was normal. These men may be started on trial of one of 5-phosphodiesterase inhibitors—see later—combined with diet, exercise, and management of existing comorbidities. Smoking cessation is critical.

However, in men with failed response to oral therapy for ED and in younger men with risks for peripheral vascular disease or neuropathy, referral for penile Doppler ultrasound and neurosensory testing may be prudent. Often patients especially younger ones want to know why they suffer from ED.

Penile Doppler ultrasound is a minimally invasive and very well-tolerated procedure. After checking blood pressure explaining risks such as need for redosing IC injection, bruise, prolonged erection, and embarrassment, patient is brought to sexual medicine laboratory when IC injection is administered in the penis to induce erection. We typically use from 5 to 10 U of Trimix and take continuous measurement of blood flow through cavernosal arteries. Patient is allowed to self-stimulate and watch adult audiovisual aids as needed. In men with severe vasculopathy the dose of medication is escalated up to 60 U; however, risk of priapism increase with higher doses. It is critical to achieve rigid erection in the sexual medicine laboratory to exclude venous leak, which is often overdiagnosed because of inadequate dosing and stimulation. We measure peak systolic velocity (PSV), end diastolic velocity (EDV), and resistive index. At the same time the length of time it takes to ejaculate or orgasm (in men with anejaculation) can be measured and the amount of force to achieve tactile stimulation to sustain erection is recorded. In men with decreased sensation to the penis, penile biothesiometry is performed and vibratory thresholds measured. Typically, once the subject achieves nonbending erection, the study is completed and the patient is observed at 15 minute intervals to assure detumescence. No patient is allowed to leave office unless his penis is flaccid. In the case of prolonged erection the 500−1000 μg of Neo-Synephrine is injected into the penis with blood pressure monitoring.

In men who present with delayed ejaculation or anejaculation the study is continued till patient achieves orgasm or at least 30 minutes passes to exclude lack of normal arousal as a reason for anejaculation.

In diabetic men who complain of anejaculation, postorgasmic urine analysis is performed to determine if subjects suffer from retrograde ejaculation.

Based on penile Doppler ultrasound, diagnosis of arteriogenic ED (PSV < 35 cm/s) or venous leak (RI < 0.75) can be established. At the same time, optimal dose of IC pharmacotherapy can be established. Often it is very relieving to the patient to show him that he can achieve erection even with IC therapy.

Men with venous leak will require typically much higher doses of IC therapy, and they are not likely to respond to oral medication initially, thus PDUS helps with directing therapy in individual patients. In men with venous leak the penile rings may help; however, they are difficult to apply in men with severe obesity and panus.

In men with neurogenic ED or ED related to low testosterone, one should consider oral 5-PDE as initial therapy. Similarly in men with mild arteriogenic ED, 5-PDE should be initial choice.

Multidisciplinary approach to treatment

Decrease in BMI, management of comorbidities, and improvement in hormonal profile through hormone replacement therapy, with medical and behavioral therapy to improve sexual function and reinforce positive behavior, should be the goals of therapy. Multidisciplinary approach, including internist, dietician, bariatric surgeon, sexual medicine expert, endocrinologist, and mental health practitioner, may be necessary for complicated patients with multiple comorbidities.

Weight management

Two approaches can be employed depending on the extent of patients' obesity, willingness to lose weight, and acceptance of medical therapy. Some practitioners will defer medical therapy to improve erectile function and focus on weight loss initially. There is no question that decrease in BMI improves erectile function, body image, and sexual desire, thus this approach can be tried in selective, motivated patients with mild-to-moderate ED; however, most of the men who present to sexual medicine specialists will present with reactive depression, poor self-esteem, marriage issues, and withdrawal from sexual activities. Thus in our practice, we strongly advocate the combination of diet and exercise with medical therapy to help with erectile function and replace testosterone in men with hypogonadism.

As sexuality plays an important role in male self-esteem, by improving sexual function we often achieve improvement in mood and dedication to weight loss [58].

High-protein, carbohydrate-reduced, low-fat diet and low-calorie diet (1000 kcal/day) over 52 weeks have similar degree in improving sexual function, sexual desire, and urinary symptoms in obese men. High-protein diet may be easier to tolerate by younger men when combined with exercise program then restrictive calorie intake diet; however, the choice of diet should be established based

on the basic metabolic rate, level of daily activities, and patients' own goals in consultation and under the supervision of dietician. Both diets help to reduce systemic inflammation [59]. Mediterranean diet helps to reduce ED [58].

Exercise has been shown to reduce elevated blood pressure and improve ED and should be combined with diet and medical therapy [60].

In severely obese patients, bariatric surgery improves erectile function, increases testosterone levels, and decreases prolactin and estradiol level, but bariatric surgery does not reverse obese-related impairment in spermatogenesis. Bariatric surgery in obese patients with severe sexual dysfunction and other comorbidities may be considered especially in patients who have contraindications or fail to respond to first- and second-line therapy for sexual dysfunction.

Pharmacological therapy

The mainstay of therapy of ED remains 5-PDEs.

5-PDE blocks the inactivation of cGMP in smooth muscles and thus improves smooth muscle relaxation.

Following 5-PDE inhibitors are approved in the United States: sildenafil (Viagra), vardenafil (Levitra and Staxyn), tadalafil (Cialis), and avanafil (Stendra). After patent for sildenafil expired in 2019, generic forms of it are widely available in the United States, but bioequivalence studies are lacking so far.

The efficacy of these agents has been established in large multinational trials in men with varied degrees of ED and broad spectrum of etiologies.

The drugs differ in their time and duration of onset, side effect profile, and effects of food on bioavailability. This is especially an important issue in men with diabetes who may suffer from gastroparesis and men with frequent snacking as in obesity. Bioavailability of sildenafil and vardenafil is significantly decreased by food so they should be taken on empty stomach. But bioavailability of tadalafil and avanafil is not affected by food so can be taken on empty stomach as well as with food. All information about pharmacological agents described next have been extracted from the latest official FDA labels for each of medications.

Sildenafil (Viagra) was the first 5-PDE approved for ED. Viagra comes in 25, 50, and 100 mg tablets. Sildenafil should be used prior to sexual activity "on-demand." Sildenafil is rapidly absorbed with median absorption of 60 minutes when taken in fasting state. High-fat food delays peak level of sildenafil by 60 minutes and mean reduction in Cmax of 29%. Although sildenafil's effect may last up to 4 hours, most of effectiveness is observed within 2 hours after oral intake. Sildenafil has been studied in men with ED and chronic stable angina limited by exercise, there was no difference in exercise-induced angina episodes between men receiving sildenafil to men on placebo. Sildenafil has dose-related impairment of color discrimination (blue/green) without change in visual acuity or intraocular pressure. 82% of men taking 100 mg of sildenafil, 74% 50 mg of sildenafil, and 63% of 25 mg reported improvement in sexual function.

Sildenafil and all 5-PDEs are contraindicated in patients taking nitrates either regularly or intermittently. Sildenafil has systemic vasodilator properties and can cause transient decrease in supine blood pressure of 8.4/5.5 in healthy volunteers. The decrease in blood pressure may be more pronounced with aortic stenosis and severely impaired autonomic control of blood pressure. Men on antihypertensive therapy may be at increased risk of hypotensive episodes; thus in these groups the therapy should be initiated at lower doses and increased slowly. Sildenafil is contraindicated in men with retinitis pigmentosa.

Important issues to discuss with all 5-PDEs are risk of developing prolonged erection (>4 hours) and priapism. Sudden loss of vision in one or both eyes may be a sign of nonarteritic anterior ischemic optic neuropathy (NAION). Most of the cases on NAION occurred in men with diabetes, HTN, coronary artery disease, hyperlipidemia, and over 50 years. Similarly, sudden decrease or loss of hearing that may be accompanied by tinnitus and dizziness has been reported as well. However, at this point, it has not been determined that the vision and hearing loss are related to the use of 5-PDE or to other factors. Sildenafil is associated with headache 16%, flushing 10%, dyspepsia 7%, and nasal congestion 4%. At 100 mg dose, 17% of patients reported dyspepsia and 11% abnormal vision.

It is critical to consider that 5-PDE should not be used in men with angina, recent acute MI, and limited cardiac reserve as normal cardiovascular function is necessary to have sexual activities.

Diabetic patients have somehow reduced response to sildenafil, as 63% of men with type II DM and 67% of men with type I DM reported improvement in erections in pulled data from 11 trials. 86% of men with hyperlipidemia reported improvement and 69% of men with HTN.

Vardenafil (Levitra) in oral form has been approved in 2003 by FDA. Levitra comes in 2.5, 5, 10, and 20 mg tablets. A 10 mg tablet taken 60 minutes prior to sexual actives is a recommended starting dose. Vardenafil as all other 5-PDEs is contraindicated in men taking nitrates, with unstable angina and hypotension.

Levitra is contraindicated in patients with congenital QT syndrome or taking class IA (quinidine, procainamide, disopyramide) or III antiarrhythmic (amiodarone, sotalol, ibutilide, dofetilide). Levitra should not be used in men with unstable angina, hypotension, uncontrolled HTN ($>170/110$ mmHg), recent history of stroke, life-threatening arrhythmias, recent myocardial infarction (<6 months), and severe cardiac failure. Levitra shares the

same warnings about prolonged erections and NAION as sildenafil. Headache in 15% of subjects, flushing in 11%, rhinitis in 9%, and dyspepsia in 4% are the most common side effects of Levitra. After oral intake of Levitra the peak concentration is noticed at median of 60 minutes in fasted state. The high-fat meals reduced in Cmax between 18% and 50%, which is of significance in obese patients who often have HFDs. Levitra shows dose-related response that in general, population of men with ED is 65% for 5 mg, 75% for 10 mg, and 80% for 20 mg compared to placebo response of 52%. However, in men with ED and DM the response was 51% at 5 mg, 64% at 10 mg, and 65% at 20 mg, thus showing a decreased response rate in patients with DM and ED by almost 15% in higher dose as compared to normal men.

Staxyn is orally disintegrable table of 10 mg of vardenafil. It is not interchangeable with 10 mg of Levitra as it achieves higher systemic exposure compared to 10 mg Levitra. In clinical studies the side effects profile was similar to Levitra, but the rate of per patient rate of achieving erection sufficient for penetration was 47% for 10 mg and 22% for placebo.

Tadalafil (Cialis) has been approved by FDA in 2003 for use in men with ED and was also approved in 2011 for treatment of men with benign prostatic hyperplasia.

Tadalafil can be used on demand at dose of 5, 10, or 20 mg or as daily 5 mg medication.

Because of its selectivity for 5-PDE, tadalafil has much less flushing and nasal congestion 2% for 5 mg and 3% for 20 mg than sildenafil or vardenafil. Headache can occur in 6% of men on daily dose of 5 mg, and 15% of men taking highest 20 mg dose. Dyspepsia is seen in 5% of men taking daily Cialis and 10% of men with 20 mg on-demand Cialis. Back pain can be observed in 6% of men taking 20 mg dose and 3% men (3%—placebo) on daily 5 mg tadalafil. 77% of men on 20 mg tadalafil versus 43% on placebo experienced improvement in ability to penetrate. 64% versus 23% on placebo reported ability to sustain erection. 57% of men with DM-related ED taking 10 mg Cialis versus 30% men taking placebo reported ability to insert. Daily 5 mg tadalafil was also successful in improving ED as 67% of men on daily tadalafil versus 37% men on placebo reported ability to insert. Similar effectiveness was seen in men with DM.

Obesity increases the frequency of *lower urinary tract symptoms and ED*. Tadalafil is the only medication in this group which is approved for men with BPH with or without ED; thus it represents good option in obese men who present with both. Bioavailability of tadalafil is not affected by food; hence, it may be a better option in general for men with obesity. However, no head-to-head study compared differences in effectiveness between three available 5-PDEs in men with delayed stomach emptying or morbid obesity.

Daily tadalafil has been now shown to improve peak systolic velocity in diabetic patients with vascular disease as compared to on-demand tadalafil [61]. Considering that daily treatment with tadalafil has recently been shown to improve endothelial function, which is impaired in men with obesity-related diseases, it seems prudent to initiate therapy with daily 5 mg tadalafil or fixed dose of 20 mg of tadalafil three times a week [62].

Avanafil (Stendra) was approved in 2012 in the United States and is considered to have unique properties of quick on quick off action. Avanafil may be taken 15 minutes before sexual activities with or without food. Avanafil comes in 50, 100, and 200 mg tablets. Recommended initial dose is 100 mg once daily as needed.

Patients with left ventricular outflow obstruction (aortic stenosis, idiopathic hypertrophic subaortic stenosis) and autonomic blood pressure dysregulation may be at risk for vasodilating effect of avanafil.

In healthy volunteers, avanafil 200 mg resulted in modest decrease in blood pressure—8 mmHg systolic and 3.3 mmHg diastolic.

All 5-PDE inhibitors are contraindicated in patients taking GC stimulators such as riociguat. CYP3A4 inhibitors taken together with avanafil may increase its level and result in significant drop in blood pressure. If therapeutically indicated, one should start at lowest dose, 50 mg. Avanafil should not be used in patients taking ritonavir because of significant increase in Cmax of avanafil. Alpha-blocker therapy together with avanafil needs to be monitored initially as combination therapy increases risks of hypotensive episodes. Headache occurred in 10.5% of patients taking 200 mg of Stendra, flushing in 4.0%, nasal congestion in 2%, and back pain in 1.1%. Studies in animals showed decreased fertility and abnormal sperm motility and morphology in animals exposed to avanafil. However, study of 181 healthy volunteers taking 100 mg of avanafil showed no effect on semen parameters. Avanafil is quickly absorbed with median Tmax of 30−45 minutes. Most of avanafil is eliminated within 8 hours from intake. Avanafil has similar effectiveness in improving erectile function as other 5-PDEs. Overall, vaginal penetration was reported by 77.3% patients on 200 mg of avanafil compared to 53.8% on placebo. Successful intercourse was reported by 57% of men on 200 mg of avanafil as compared to 27% of men taking placebo. However, for men with DM, 63% were able to penetrate vaginally (42% placebo) and 40% had successful intercourse (20.5% placebo).

All 5-PDEs share similar contraindications described earlier.

Penile intracavernosal injection therapy

Subjects who fail oral therapy may benefit from *penile IC injection therapy* [63]. Self-administered penile injection

therapy is well tolerated and may be more successful in diabetic men with severe ED as compared to oral agents [64]. IC therapy can be started with premixed Caverject (prostaglandin E1). PGE1 relaxes smooth muscles in penis through EP receptor [65]. *Caverject* comes in prefilled vials of 5, 10, 20, and 40 μg to be used with self-injector or single-use syringe, which can deliver from 6 to 20 μg of medication. Side effects of Caverject are penile pain which can occur in 37% of patients, penile fibrosis in 3%, prolonged erection in 4%, and injection site hematoma in 3%. Priapism has been reported in 0.4% subjects according to FDA label. Over 72% of men with diabetes responded to Caverject. Many practices, including ours, use multidrug combination of vasoactive agents for IC injections, which may decrease pain and improve efficacy as compared to prostaglandin E1 [66]. Most commonly used preparations are *Trimix* (papaverine 30 mg, phentolamine 1 mg, and prostaglandin E1 10 μg/1 mL) and *super-Trimix* (papaverine 30 mg, phentolamine 2 mg, and prostaglandin E1 20 μg/1 mL). Typically, we use 0.05−0.1 mL of Trimix to start. It is critical to remember that all forms of IC therapy have to be initiated and dose titrated in physician office prior to prescribing an adequate dose.

Penile prosthesis

Penile prosthesis can be considered in men who failed injection therapy; however, it is more important for obese men that they should first consider weight loss and aggressive treatment of hypercholesterolemia and DM prior to penile prosthesis as once placed, it has to remain in the penis to prevent scarring and dramatic penis shortening. Obesity is associated with decreased satisfaction with penile prosthesis [67]. Poorly controlled diabetes is associated with increased risk of penile prosthesis infections [68]. Long-term follow-up studies showed that at 5 years, 1:10 penile prosthesis has to be replaced [69].

Testosterone replacement therapy

It is well established that obese men suffer from hypogonadism but it is less clear if testosterone replacement therapy (TRT) should be considered in all obese men [70]. A significant amount of data exists which supports combining weight loss strategy with the use of TRT [71]. TRT may decrease BMI and improve metabolic markers of cardiovascular risks [72,73].

TRT may be administered using injectable forms of therapy—such as testosterone enanthate or cypionate, testosterone pellets, or topical forms of replacement. Injectable testosterone is typically administered as intramuscular injection at 2 weeks intervals at 200−300 mg of depot-testosterone. Topical preparations can be applied to underarm (Axrion), shoulders (Androgel and Testim), and inner tight (Fortesta).

Goal of therapy is to achieve normal testosterone levels. Based on recent large data analysis of patients enrolled in MrOS study in Sweden, the cardioprotective effect of testosterone was observed if levels were above 550 ng/dL; thus we typically set goal of therapy in upper normal range [54]. We use exclusively topical preparations because of significantly less risk of polycythemia as compared to injectable agents.

TRT may have positive metabolic effect in obese men but also improve their sexual drive, ejaculatory volume, and orgasmic function and improve patients' quality of life. Further studies are needed to prove the positive long-term benefits of TRT in obese men. In men who failed to improve TT despite use of adequate dose of TRT, aromatase inhibitors such as anastrozole 1 mg daily may be used for set amount of time (6−12 months) to overcome excessive conversion of testosterone into E2 seen in many obese men [74,75].

Prevention

A longitudinal study of close to 4 million patients in the United Kingdom showed that obesity is diagnosed at young people, 20−29 years, and ED diagnosis increases dramatically in men, 40−59 years. Thus there seems to be a window of opportunity early on to manage obesity in young men. Perhaps, disseminating information that obesity today may lead to loss of erectile function in future may be an impetus for younger men to lose weight.

In summary, sexual dysfunction is common in obese men. The risk of ED is related to obesity-related comorbidities such as hypogonadism, peripheral vascular disease, hypercholesterolemia, and diabetes rather than simple increase in BMI. Reduction in BMI combined with successful treatment of sexual dysfunction improves the quality of life and has a positive motivator effect on compliance with control of comorbidities. Further placebo control studies are needed to establish a role of multimodal therapy in obese men with sexual dysfunction.

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